

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Confirmation No.: 1470

Chyi-Cheng CHEN *et al.*

Group Art Unit: 1611

Application Serial No.: 09/726,880

Examiner: CHANNAVAJJALA, L. S.

Filed: November 30, 2000

Attorney Docket No.: 2921268-001000

For: A VITAMIN POWDER COMPOSITION AND METHOD OF MAKING

**APPEAL UNDER 35 U.S.C. § 134**

## TABLE OF CONTENTS

I.	REAL PARTY IN INTEREST .....	4
II.	RELATED APPEALS AND INTERFERENCES .....	4
III.	STATUS OF CLAIMS.....	4
IV.	STATUS OF AMENDMENTS .....	5
V.	SUMMARY OF CLAIMED SUBJECT MATTER .....	5
A.	CLAIM 3 .....	5
C.	CLAIM 5 .....	5
D.	CLAIM 6 .....	5
E.	CLAIM 7 .....	5
F.	CLAIM 8 .....	6
G.	CLAIM 9 .....	6
H.	CLAIM 10 .....	6
I.	CLAIM 11 .....	6
J.	CLAIM 12 .....	6
K.	CLAIM 13 .....	6
L.	CLAIM 14 .....	7
M.	CLAIM 17 .....	7
N.	CLAIM 28 .....	7
O.	CLAIM 29 .....	7
P.	CLAIM 30 .....	8
Q.	CLAIM 31 .....	8
R.	CLAIM 33 .....	8
S.	CLAIM 34 .....	8
T.	CLAIM 35 .....	8
U.	CLAIM 36 .....	8
VI.	GROUND OF REJECTION TO BE REVIEWED ON APPEAL .....	9
VII.	ARGUMENTS.....	9
A.	CLAIMS 3-14, 17, 28-31, AND 33-36 ARE NOT OBVIOUS OVER EITHER AUWETER IN VIEW OF LORANT OR AUWETER AND STEIN IN VIEW OF LORANT .....	9
1.	<i>Failure to Establish a Prima Facie Case of Obviousness for Claim 36</i> .....	9
a.	Instant claimed average diameter of about 80 to about 120 nm is integral to optical clarity and appearing transparent and/or translucent in solution upon addition to a clear liquid.....	10
b.	No reason to turn to Auweter and Stein as their goals are to invent better COLORING compositions, not the instant desired goal of optical clarity .....	11
c.	Auweter and Stein goals of inventing better COLORING compositions and disclosed properties and particle size would produce turbid and colored solution, and not achieve the instant desired clarity .....	12
d.	The processes of Auweter and Stein does not provide any guidance as to how to make the presently claimed composition .....	14
e.	No reason to modify Auweter or Stein with Lorant.....	15
2.	<i>Claim 3 is Independently Patentable</i> .....	17
3.	<i>Claim 4 is Independently Patentable</i> .....	17
4.	<i>Claim 5 is Independently Patentable</i> .....	17
5.	<i>Claim 6 is Independently Patentable</i> .....	18
6.	<i>Claim 7 is Independently Patentable</i> .....	18
7.	<i>Claim 8 is Independently Patentable</i> .....	19
8.	<i>Claim 9 is Independently Patentable</i> .....	19
9.	<i>Claim 10 is Independently Patentable</i> .....	20
10.	<i>Claim 11 is Independently Patentable</i> .....	20
11.	<i>Claim 12 is Independently Patentable</i> .....	21

12. <i>Claim 13 is Independently Patentable</i> .....	22
13. <i>Claim 14 is Independently Patentable</i> .....	22
14. <i>Claim 17 is Independently Patentable</i> .....	23
15. <i>Claim 28 is Independently Patentable</i> .....	23
16. <i>Claim 29 is Independently Patentable</i> .....	24
17. <i>Claim 30 is Independently Patentable</i> .....	26
18. <i>Claim 31 is Independently Patentable</i> .....	26
19. <i>Claim 33 is Independently Patentable</i> .....	27
20. <i>Claim 34 is Independently Patentable</i> .....	28
21. <i>Claim 35 is Independently Patentable</i> .....	29
B. CLAIM 36 IS NOT OBVIOUS OVER EITHER AUWETER IN VIEW OF LORANT AND CANNALONGA OR AUWETER AND STEIN IN VIEW OF LORANT AND CANNALONGA .....	30
VIII. CONCLUSION.....	31
IX. CLAIMS APPENDIX .....	32
X. EVIDENCE APPENDIX .....	35
XI. RELATED PROCEEDINGS APPENDIX.....	36

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**APPEAL BRIEF**

**I. Real Party in Interest**

The real party in interest is DSM Nutritional Products, Inc., Parsippany, New Jersey, by virtue of the Assignment recorded at reel/frame number 015452/0973.

**II. Related Appeals and Interferences**

Appellants' are not aware of any prior or pending appeal, judicial proceeding, or interference, which may be related to, directly affect or be directly affected by, or have a bearing on the Board's decision in the pending appeal.

**III. Status of Claims**

Claims 3-14, 17, 28-31, and 33-36 are currently pending and are under appeal. Claims 3-14, 17, 28-31, and 33-36 stand rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over either US 5,968,251 to Auweter ("Auweter") in view of US 5,952,395 to Lorant *et al.* ("Lorant") or Auweter and EP 937412 ("Stein") in view of Lorant.

Claim 36 stand rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over Auweter in view of Lorant and US 2,756,177 to Cannalunga ("Cannalunga") or Auweter and Stein in view of Lorant and Cannalunga.

#### **IV. Status of Amendments**

As set forth in the Office Action dated June 22, 2011, the Examiner entered the amendments and arguments submitted in the Request for Continued Examination (RCE) dated May 2, 2011.

#### **V. Summary of Claimed Subject Matter**

Below is a summary of the claims discussed herein with reference to the specification:

##### **A. Claim 3**

Dependent Claim 3 recites: The powder composition according to claim 36, wherein the polysaccharide gum is isolated from the group consisting of plants, animals and microbial sources (Specification at page 8, ll. 16-19)

##### **B. Claim 4**

Dependent claim 4 recites: The powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of exudate gums, seaweed gums, seed gums, microbial gums, and mixtures thereof (Specification at page 8, ll. 19-20).

##### **C. Claim 5**

Dependent claim 5 recites: The powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of gum arabic, flaxseed gum, ghatti gum, tamarind gum, arabinogalactan, and mixtures thereof (Specification at page 8, ll. 24-25).

##### **D. Claim 6**

Dependent claim 6 recites: The powder composition according to claim 5 wherein the polysaccharide gum is gum arabic (Specification at page 8, ll. 26).

##### **E. Claim 7**

Dependent claim 7 recites: The powder composition according to claim 36 wherein the protein is isolated from a plant or an animal source (Specification at page 9, ll. 20-21).

**F. Claim 8**

Dependent Claim 8 recites: The powder composition according to claim 7 wherein the protein is selected from the group consisting of sunflower proteins, soy-bean proteins, cotton seed proteins, peanut proteins, rape seed proteins, milk proteins, blood proteins, egg proteins, gelatine, crosslinked gelatine, and mixtures thereof (Specification at page 9, ll. 21-24).

**G. Claim 9**

Dependent claim 9 recites: The powder composition according to claim 8 wherein the protein is gelatine (Specification at page 9, ll. 24).

**H. Claim 10**

Dependent claim 10 recites: The powder composition according to claim 36 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E or its esters, vitamin A or its esters, vitamin K (phytomenadione), vitamin D3 (cholecalciferol), and mixtures thereof (Specification at page 10, ll. 19-21).

**I. Claim 11**

Dependent claim 11 recites: The powder composition according to claim 10 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E acetate, vitamin A acetate, vitamin A palmitate, and mixtures thereof (Specification at page 9, ll. 19-20).

**J. Claim 12**

Dependent claim 12 recites: The powder composition according to claim 36 wherein the ratio of fat-soluble vitamin to matrix component is from about 1:99 to about 3:1 (Specification at page 12, ll. 3-5).

**K. Claim 13**

Dependent claim 13 recites: The powder composition according to claim 12 wherein the ratio of fat-soluble vitamin to matrix component is from about 1:8 to about 1:1 (Specification at page 12, ll. 7-8).

**L. Claim 14**

Dependent claim 14 recites: The powder composition according to claim 36 wherein the composition comprises from about 60 to 85% by weight of a matrix component, based on the total weight of all the components present in the composition (Specification at page 12, ll. 5-7).

**M. Claim 17**

Dependent claim 17 recites: The powder composition according to claim 36, which is formed into a tablet (Specification at page 12, ln. 23).

**N. Claim 28**

Dependent claim 28 recites: The powder composition according to claim 36 wherein the emulsion-forming composition is an oil-in water emulsion-forming composition (Specification at page 7, ll. 9-11).

**O. Claim 29**

Independent claim 29 recites: A powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets (Specification at page 2, ll. 16-21), wherein the powder composition is produced by a process comprising:

(a) combining water with a matrix component selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, to form a solution (Specification at page 3, ll. 19-20);

(b) adding a fat-soluble vitamin to the solution to form a crude emulsion (Specification at page 3, ll. 19-20), wherein the fat-soluble vitamin is added in an amount to provide from about 10% to about 30% by weight fat-soluble vitamin in the powder composition ((Specification at page 25, ll. 18-21);

(c) emulsifying the crude emulsion at a temperature of from about 5° C to about 75° C at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080

bar) (Specification at page 3, ll. 22-24), to obtain an emulsion in which the droplets have an average diameter of about 80 to about 120 nm (Specification at page 4, ll. 24-25); and

(d) drying the emulsion to obtain the powder composition (Specification at page 3, ln. 26).

**P. Claim 30**

Dependent claim 30 recites: The powder composition according to claim 29, wherein the crude emulsion formed has a solids content of from about 30%(wt) to about 50%(wt) (Specification at page 22, ll. 14-16).

**Q. Claim 31**

Dependent claim 31 recites: The powder composition according to claim 29, further comprising mixing the crude emulsion prior to emulsifying, to provide a crude emulsion having droplets which are about 1500 nm or less (Specification at page 22, ll. 17-18).

**R. Claim 33**

Dependent claim 33 recites: The powder composition according to claim 29, wherein the emulsifying occurs at a pressure of from about 20,000 psi (about 1,360 bar) to about 35,000 psi (about 2380 bar) (Specification at page 26, ll. 21-22).

**S. Claim 34**

Dependent claim 34 recites: The powder composition according to claim 29, wherein the emulsifying occurs at a pressure of about 25,000 psi (about 1,700 bar) (Specification at page 22, ln. 21).

**T. Claim 35**

Dependent claim 35 recites: The powder composition according to claim 29 wherein the emulsion-forming composition is an oil-in water emulsion-forming composition (Specification at page 9, ll. 28-29).

**U. Claim 36**

Independent claim 36 recites: A powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide



gums, a protein, a mixture of proteins, and mixtures thereof (Specification at page 2, ll. 16-20), wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets having an average diameter of about 80 to about 120 nanometers (nm) (Specification at page 4, ll. 24-25) and wherein the fat-soluble vitamin is present in the powder composition in the amount of from about 10% to about 30% by weight (Specification at page 25, ll. 18-21) and wherein the composition has a moisture content of about 1 to 4% by weight (Specification at page 11, ll. 19-20 and ll. 24-25).

**VI. Grounds of Rejection to be Reviewed on Appeal**

(1) Whether claims 3-14, 17, 28-31, and 33-36 are properly rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over either Auweter in view of Lorant or Auweter and Stein in view of Lorant.

(2) Whether claim 36 is properly rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over either Auweter in view of Lorant and Cannalonga or Auweter and Stein in view of Lorant and Cannalonga

**VII. Arguments**

Claims 3-14, 17, 28-31, and 33-36 are rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over either Auweter in view of Lorant or Auweter and Stein in view of Lorant. Claim 36 is rejected under 35 U.S.C. § 103(a) as being allegedly unpatentable over either Auweter in view of Lorant and Cannalonga or Auweter and Stein in view of Lorant and Cannalonga. Appellants respectfully traverse the rejections for at least the following reasons.

**A. Claims 3-14, 17, 28-31, and 33-36 are not obvious over either Auweter in view of Lorant or Auweter and Stein in view of Lorant**

**1. Failure to Establish a *Prima Facie* Case of Obviousness for Claim 36**

In rejecting Claim 36, the Examiner asserts that Auweter teaches cold water dispersible powders comprising fat soluble vitamins such as carotenoids prepared by the method described in abstract and col. 2, ll. 27-46. Office Action dated June 22, 2011, page 3. The Examiner asserts

that for the protective colloids, Auweter teaches the claimed proteins such as fish gelatin, vegetable proteins, and also gum such as gum Arabic, and also teaches a 0.5 to 20% carotenoids and 10-50% by weight of a protective colloid. *Id.* The Examiner acknowledges that Auweter does not exemplify any compositions with the claimed gums or proteins. *Id.* The Examiner also acknowledges that Auweter teaches particles of 200 nm size, but does not teach the claimed particle size of 80-120 nm. *Id.*

The Examiner asserts that Stein teaches for the colloids, gelatin, starch, gums, pectin, etc, and particle size such as 213 nm, 225 nm, or 400nm. *Id.* at page 4. However, the Examiner acknowledges that Stein also does not teach the claimed particle size of 80-120 nm. *Id.*

The Examiner finally asserts that Lorant teaches nanoparticle sizes in the range of 30-200 nm. *Id.* Thus, the Examiner reasons that it would have been obvious to one of ordinary skill in the art to prepare the powders of Auweter in nanoparticle size range suggested by Stein and further incorporate the size ranges of Lorant. *Id.*

Appellants respectfully traverse. It would not have been obvious for one of ordinary skill in the art to combine Auweter, Stein, and Lorant to achieve the instant claimed powder composition as the disclosures of Auweter and Stein do not suggest formulations having the claimed droplet size having an average diameter of about 80 to about 120 nm, which as discussed below, have optical clarity and which appear transparent and/or translucent in an otherwise clear solution. Auweter and Stein are directed to the goal of producing better coloring compositions, and their properties and disclosed particle size of about 200 nm would produce a turbid and colored composition, and Lorant does not remedy these deficiencies.

**a. Instant claimed average diameter of about 80 to about 120 nm is integral to optical clarity and appearing transparent and/or translucent in solution upon addition to a clear liquid**

As explained in the Declaration of Dr. Bruno Leuenberger submitted herewith (originally submitted with the RCE dated December 7, 2009), the claimed powder composition achieves optical clarity. See Declaration ¶¶ 5 and 6 and instant Specification, page 7, ll. 1-6.

As stated in the Specification, "[f]at soluble vitamins for supplementation...when added to beverages, will tend to increase the visible turbidity," and "one object of the present invention is to provide a powder composition of fat-soluble vitamins which can be added to

beverages...preferably without affecting the optical clarity of the beverage." Instant Specification, page 1, ll. 15-17 and ll. 24-26.

The Specification goes on to state that "optical clarity may be estimated by a visual comparison, such that if there is no significant visible added turbidity, the liquid has retained its optical clarity." Instant Specification, page 6, ll. 5-6. Optical clarity (turbidity) can be accurately measured by a turbidimeter, and the amount of reflected light is directly proportional to the degree of turbidity. *Id.* at page 6, ll. 6-7, ll. 17-18.

Turbidity is very low for particles having smaller diameters, and turbidity approaches zero for particle diameters below 200. *See* Declaration ¶ 21, and Instant Specification, page 6, ll. 3-11. Furthermore, at a particle size of about 200 nm, particles are at a transitional point between the invisible and visible ranges of the spectrum, and particles having an average diameter in a range from about 80 to about 120 nm are in the invisible size range. *Id.* at ¶ 22. The Specification also states that "with regard to obtaining a powder containing droplets of a size which provide an acceptable turbidity for the desired fortification level, the droplet size resulting from addition of the powder is the most important variable." *See* instant Specification, page 26, ll. 12-14.

Thus, based on the foregoing, Dr. Leuenberger states the claimed particles having an average diameter of about 80 to about 120 nm are small enough to allow sufficient transmission of light such that a clear liquid in which a powder composition having such particles is added appears as transparent and/or translucent solution which is essentially free from turbidity, and the claimed particle size range is integral to the favorable aspects achieved above. *Id.*

It is apparent from the Declaration of Dr. Leuenberger and the Specification that the properties of optical clarity and appearing transparent and/or translucent in solution upon addition to a clear liquid are advantageous properties that result from the powder composition as claimed.

**b. No reason to turn to Auweter and Stein as their goals are to invent better COLORING compositions, not the instant desired goal of optical clarity**

Auweter's objective is to prepare better coloring compositions using carotenoid preparations to overcome the previous "poor coloring results," and discloses that "the

preparations according to the invention are... outstandingly suitable as coloring agents for food, especially soft drinks." *Id.* at col. 2, ll. 21-26, and col. 5, ll. 35-37. Examples 1-2 of Auweter also disclose that the preparations according to the invention formed "clear yellow dispersions" and "dispersion with an orange hue." *Id.* at Examples 1-2, col. 6. Auweter also discloses that the process variant results in "the precipitated active substance particles after heating...with a diameter of typically, 200 nm." *Id.* at col. 3, ll. 51-57. There is no disclosure or suggestion in Auweter of any particle size of precipitated active substance that does not have at least a 200 nm diameter.

Stein also discloses a "continuous process for converting carotenoids, retinoids, or natural colourants into finely divided pulverous forms which are particularly required for colouring foodstuff and animal feeds." *See* Stein, [0001]. Examples 1-5 of Stein discloses particle sizes of 196 nm- 240 nm, and solutions with intensive yellow, red, or cherry-red colorations.

Based on the foregoing, Auweter and Stein provide no motivation for one of ordinary skill in the art to achieve the claimed powder composition. In fact, the goals of Auweter and Stein to invent better coloring agents are antithetical to the claimed goal of achieving optical clarity and a transparent and/or translucent solution upon addition to a clear liquid, and one of ordinary skill in the art would not be motivated to look at technologies directed to better coloring effects for inventive compositions directed to optical clarity.

**c. Auweter and Stein goals of inventing better COLORING compositions and disclosed properties and particle size would produce turbid and colored solution, and not achieve the instant desired clarity**

The physiochemical properties of carotenes such as  $\beta$ -carotene and instant claimed fat-soluble vitamins, such as  $\alpha$ -tocopherol, are known to differ markedly. *See* Leuenberger Declaration, ¶ 23. As indicated in the entry for  $\beta$ -carotene in the Merck Index (Exhibit D),  $\beta$ -carotene is an insoluble solid compound, and the use indicated in the Merck Index is as a "[y]ellow coloring agent for foods." Declaration, ¶ 23 and Exhibit D, page 1854, ll. 15, 19, and 26.  $\alpha$ -tocopherol, however, is a liquid oil. Declaration, ¶ 23. Thus, the physiochemical properties of  $\beta$ -carotene and instant claimed fat-soluble vitamins differ in significant ways that affect the goals in formulation, and in looking to formulate a liquid oil to achieve a powder composition having optical clarity, one of ordinary skill in the art would not have looked to technologies for

formulating a solid, insoluble, colored compound which are for preparing compositions directed to better coloring agents. *Id.*

In fact, the use of  $\beta$ -carotene in Auweter is antithetical to the claimed powder composition which achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid. *Id.* at ¶ 27. Dr. Leuenberger asserts that Auweter provides a  $\beta$ -carotene powder composition that renders  $\beta$ -carotene, which is otherwise insoluble, dispersible as fine particles in cold water, and the dispersion disclosed by Auweter is a solid in liquid dispersion stabilized with colloids. *Id.* at ¶ 25. This is markedly different from the claimed composition that provides solid droplets of a fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition, where the emulsion-forming composition has sufficient emulsifying properties in an oil-in-water context to emulsify the oil into a fine dispersion in an aqueous medium and forms a stable emulsion of the claimed droplet size. *Id.* at ¶ 26.

Thus, Dr. Leueuberger asserts that in view of the foregoing characteristics of Auweter's composition and Auweter's disclosed particle size of about 200 nm, when added to a previously clear liquid, it would produce a turbid and colored solution, which is consistent with Auweter's stated goal of providing better coloring agents. *Id.* at ¶ 25.

Also asserted in the Stein Declaration (originally submitted with the response dated March 28, 2005), Mr. Hermann Stein, who is the inventor of Stein cited by the Examiner, declares that "[d]uring the research that led to the invention disclosed in Stein, my co-inventors and I, using the knowledge available at the time, attempted to produce the smallest possible particle size." See Stein Declaration, ¶ 6. Stein goes on to assert that "[a]s Example 5 shows, at the time, at best we could produce particle sizes of about 196 nm." *Id.* at

¶ 7. Stein declares that "one of skill in the art at the time of the above-captioned invention familiar with the disclosures of Stein could not have produced particles of the presently claimed size," and "one could not have predicted that the process of the present invention would produce significantly smaller particle sizes than the methods of Stein." *Id.* at ¶¶ 7, 8.

Thus, whereas Auweter's  $\beta$ -carotene powder composition and Stein's carotenoid preparation having particle sizes of about 200 nm and 196 nm, which is visible to the eye, would produce a turbid and colored solution, the claimed powder composition having solid droplets of fat soluble

vitamin with an average diameter of about 80 to about 120 nm, which are not in the visible range, achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid. Thus, one of ordinary skill in the art would have no motivation to look to inventions directed to better coloring effects with properties and particle sizes that would produce turbid and colored solutions, for inventive compositions directed to optical clarity.

Thus, in looking to formulate a liquid oil to achieve a powder composition having optical clarity, one of ordinary skill in the art would not have looked to such technologies for formulating a solid, insoluble, colored compound for preparing compositions directed to better coloring agents.

**d. The processes of Auweter and Stein does not provide any guidance as to how to make the presently claimed composition**

Further, Dr. Leuenberger indicates that Auweter discloses a process that differs from the process used in making the claimed powder composition, and does not provide any guidance as to how the presently claimed composition can be produced. Leuenberger Declaration, ¶ 28.

As disclosed in the Specification, "[t]he polysaccharide gum and/or the protein, as used in this invention both have sufficient emulsifying properties. This means that they have sufficient emulsifying properties in an oil-in-water context to emulsify the oil into a fine dispersion in an aqueous medium and are capable of forming a stable emulsion of a desired droplet size...under conditions of high pressure homogenization." *See* instant Specification, page 7, ll. 8-13. High pressure means a pressure of about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar). *Id.*

Auweter discloses how to make a solid in liquid dispersion of fine particles of  $\beta$ -carotene stabilized with colloids, and does not disclose a process using an emulsion-forming composition that has sufficient emulsifying properties in an oil in water context to form a stable emulsion of the claimed droplet size. Leuenberger Declaration, ¶ 28. Moreover, Auweter's disclosure of the use of atmospheric or superatmospheric pressure, e.g., in the range of 20 to 80 bar, preferably 30 to 60 bar, to produce a cold-water dispersible powder, provides no indication, or expectation of success, of using a high-pressure homogenization process at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar), as claimed. *Id.* and Auweter, Col. 2, ll. 59-62. The range pressure indicated for Auweter is much less than the range indicated for the

instant invention, and application of the instantly claimed pressure range would have been a matter beyond routine experimentation.

Also, Dr. Leuenberger's Declaration asserts that even if one were to consider using greater pressure, although Auweter provides no suggestion of this, the literature indicates that pressure is not a result-effective variable in relation to the diameter of particles in an emulsion. *See Declaration*, ¶ 29.

As for Stein, Dr. Leuenberger states that the process of Stein is similar to the process of Auweter in achieving their mutual goal of preparing carotenoid powder compositions for use as colorants. *Id.* at ¶ 30. The principle difference between the processes is that Auweter discloses the use of a water-miscible organic solvent, whereas Stein discloses the use of a water immiscible organic solvent in the first step of forming a suspension of a carotenoid. *Id.*

Thus, Dr. Leuenberger expects that the processes of Stein and Auweter would produce carotenoid particle sizes in a comparable range of about 200 nm, which is much higher than the claimed range of about 80 to about 120 nm. *Id.* Thus, Auweter provides no motivation or guidance for one skilled in the art to achieve the claimed powder composition. *Id.* at ¶ 27.

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Thus, based on the above, one of ordinary skill in the art would not have turned to the teachings of Auweter or Stein when inventing new powder compositions with optical clarity, and could not have predicted from Auweter alone or in view of Stein that the claimed powder composition could be produced having a fat soluble vitamin in the form of solid droplets having an average diameter of about 80 to about 120 nm, and which achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid.

**e. No reason to modify Auweter or Stein with Lorant**

As asserted above, one of ordinary skill would not have looked to either Auweter or Stein for new compositions with optical clarity. There is also no reason to modify Auweter or Stein with Lorant, and even if one did, Lorant does not remedy the deficiencies of Auweter or Stein.

The Examiner fails to set forth a legitimate rationale for modifying the carotenoid preparations of Auweter and Stein, with the cosmetic or dermatological composition in the form of a gelled ultrafine oil-in-water emulsion of Lorant, to achieve the composition of claim 36.

Both Auweter and Stein are directed to carotenoid preparations directed to producing better coloring effects in food. *See* Auweter, col. 2, ll. 21-26 and Stein, Examples 1-5. In contrast, Lorant is directed to a gelled ultrafine oil-in-water emulsion capable of being obtained by phase inversion, where gelling of this type of emulsion improves its stability for better cosmetic or dermatological compositions. *See* Lorant, col. 1, ll. 8-13, col. 2, ll. 15-19, and ll. 36-38.

Thus, one of ordinary skill in the art would have no motivation to combine Auweter, Stein, and Lorant, as one would not look to a non-edible gelled ultrafine oil-in-water emulsion which improve the stability of emulsions used in cosmetic or dermatological compositions, when inventing powders directed to better food coloring effects, or inventing the instantly claimed dry powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition.

Also, as stated above, the goals of Auweter and Stein are to produce a turbid and colored solution for food coloring effects, and this characteristic of the compositions of Auweter and Stein is partly due to their particle size ranges of about 200 nm or greater, while the instantly claimed average size range of about 80 to about 120 nm is integral to optical clarity and appearing transparent and/or translucent in solution upon addition to a clear liquid. Thus, one of ordinary skill in the art would not want to incorporate the size ranges of Lorant (30-200 nm) into the teachings of Auweter and Stein because that incorporation would likely produce optically clear liquid, which would defeat the purposes of Auweter and Stein of producing colored solutions.

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For the above reasons, claim 36 would not have been rendered obvious over Auweter in view of Lorant, or Auweter and Stein in view of Lorant. Claims 3-14, 17, and 28 variously depend from claim 36 and, thus, also would not have been rendered obvious. Accordingly, Appellants respectfully request reconsideration and withdrawal of the 103(a) rejection.



**2. Claim 3 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 3 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36, wherein the polysaccharide gum is isolated from the group consisting of plants, animals and microbial sources." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 3 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**3. Claim 4 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 4 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of exudate gums, seaweed gums, seed gums, microbial gums, and mixtures thereof." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 4 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**4. Claim 5 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 5 and submit that this claim is separately patentable.

The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of gum Arabic, flaxseed gum, ghatti gum, tamarind gum, arabinogalactan, and mixtures thereof." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 5 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**5. Claim 6 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 6 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 5 wherein the polysaccharide gum is gum Arabic." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 6 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**6. Claim 7 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 7 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36 wherein the protein is isolated from a plant or an animal source." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 7 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**7. Claim 8 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 8 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 7 wherein the protein is selected from the group consisting of sunflower proteins, soy-bean proteins, cotton seed proteins, peanut proteins, rape seed proteins, milk proteins, blood proteins, egg proteins, gelatine, crosslinked gelatine, and mixtures thereof." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 8 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**8. Claim 9 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 9 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 8 wherein the protein is gelatine." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 9 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**9. Claim 10 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 10 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E or its esters, vitamin A or its esters, vitamin K (phytomenadione), vitamin D3 (cholecalciferol), and mixtures thereof."

The Examiner acknowledges that Auweter teaches carotenoids esters, and not the claimed vitamins of claims 10-11, and has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 10 obvious. Office Action dated June 22, 2011, page 3.

Also, as asserted above, the physiochemical properties of carotenes such as  $\beta$ -carotene and instant claimed fat-soluble vitamins, such as  $\alpha$ -tocopherol, are known to differ markedly. *See* Leuenberger Declaration, ¶ 23. As indicated in the entry for  $\beta$ -carotene in the Merck Index (Exhibit D),  $\beta$ -carotene is an insoluble solid compound, and the use indicated in the Merck Index is as a "[y]ellow coloring agent for foods." Declaration, ¶ 23 and Exhibit D, page 1854, ll. 15, 19, and 26.  $\alpha$ -tocopherol, however, is a liquid oil. Declaration, ¶ 23. Thus, the physiochemical properties of  $\beta$ -carotene and instant claimed fat-soluble vitamins differ in significant ways that affect the goals in formulation, and in looking to formulate a liquid oil to achieve a powder composition having optical clarity, one of ordinary skill in the art would not have looked to technologies for formulating a solid, insoluble, colored compound which are for preparing compositions directed to better coloring agents. *Id.*

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**10. Claim 11 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 11 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 10 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E acetate, vitamin A acetate, Vitamin A palmitate, and mixtures thereof."

The Examiner acknowledges that Auweter teaches carotenoids esters, and not the claimed vitamins of claims 10-11, and has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 11 obvious. Office Action dated June 22, 2011, page 3.

Also, as asserted above, the physiochemical properties of carotenes such as  $\beta$ -carotene and instant claimed fat-soluble vitamins, such as  $\alpha$ -tocopherol, are known to differ markedly. *See* Leuenberger Declaration, ¶ 23. As indicated in the entry for  $\beta$ -carotene in the Merck Index (Exhibit D),  $\beta$ -carotene is an insoluble solid compound, and the use indicated in the Merck Index is as a "[y]ellow coloring agent for foods." Declaration, ¶ 23 and Exhibit D, page 1854, ll. 15, 19, and 26.  $\alpha$ -tocopherol, however, is a liquid oil. Declaration, ¶ 23. Thus, the physiochemical properties of  $\beta$ -carotene and instant claimed fat-soluble vitamins differ in significant ways that affect the goals in formulation, and in looking to formulate a liquid oil to achieve a powder composition having optical clarity, one of ordinary skill in the art would not have looked to technologies for formulating a solid, insoluble, colored compound which are for preparing compositions directed to better coloring agents. *Id.*

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

#### **11. Claim 12 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 12 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36 wherein the ratio of fat-soluble

vitamin to matrix component is from about 1:99 to about 3:1.” The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 12 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

## **12. Claim 13 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 13 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of “[t]he powder composition according to claim 12 wherein the ratio of fat-soluble vitamin to matrix component is from about 1:8 to about 1:1.” The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 13 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

## **13. Claim 14 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 14 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of “[t]he powder composition according to claim 36 wherein the composition comprises from about 60 to 85% by weight of a matrix component, based on the total weight of all the components present in the composition.” The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 14 obvious.

In fact, the Examiner asserts that Auweter teaches 10-50% by weight of a protective colloid (which the Examiner equates with the matrix component), which would fall outside the range of the matrix component as asserted by claim 14. Office Action dated June 22, 2011, page 3.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**14. Claim 17 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 17 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36, which is formed into a tablet."

The Examiner asserts that Auweter teaches the carotenoids powders for food compositions but not tablet preparations. Office Action dated June 22, 2011, page 3. However, the Examiner goes on to assert that preparing an appropriate form of the composition such as powder or solid tablet or liquid depending on the food preparation would have been within the scope of a skilled artisan. *Id.* However, the Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 17 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**15. Claim 28 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 28 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 36 wherein the emulsion-forming composition is an oil-in water

emulsion-forming composition.” The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 28 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**16. Claim 29 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over independent claim 29 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[a] powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets, wherein the powder composition is produced by a process comprising:

(a) combining water with a matrix component selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, to form a solution;

(b) adding a fat-soluble vitamin to the solution to form a crude emulsion, wherein the fat-soluble vitamin is added in an amount to provide from about 10% to about 30% by weight fat-soluble vitamin in the powder composition;

(c) emulsifying the crude emulsion at a temperature of from about 5° C to about 75° C at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar), to obtain an emulsion in which the droplets have an average diameter of about 80 to about 120 nm; and

(d) drying the emulsion to obtain the powder composition.”



The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 29 obvious.

Also, Dr. Leunenberger explains in his Declaration that Auweter discloses a process that differs from the process used in making the claimed powder composition, and does not provide any guidance as to how the presently claimed composition can be produced. Declaration, ¶ 28.

Auweter discloses how to make a solid in liquid dispersion of fine particles of  $\beta$ -carotene stabilized with colloids, and does not disclose a process using an emulsion-forming composition that has sufficient emulsifying properties in an oil in water context to form a stable emulsion of the claimed droplet size. *Id.*

Moreover, Auweter's disclosure of the use of atmospheric or superatmospheric pressure, e.g., in the range of 20 to 80 bar, preferably 30 to 60 bar, to produce a cold-water dispersible powder, provides no indication, or expectation of success, of using a high-pressure homogenization process at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar), as disclosed. *Id.* and Auweter, Col. 2, ll. 59-62. The range pressure indicated for Auweter is much less than the range indicated for the instant invention, and application of the instantly claimed pressure range would have been a matter beyond routine experimentation.

Also, Dr. Leunenberger's Declaration describes that even if one were to consider using greater pressure, although Auweter provides no suggestion of this, the literature indicates that pressure is not a result-effective variable in relation to the diameter of particles in an emulsion. *See Declaration*, ¶ 29.

As for Stein, Dr. Leunenberger states that the process of Stein is similar to the process of Auweter in achieving their mutual goal of preparing carotenoid powder compositions for use as colorants. *Id.* at ¶ 30. The principle difference between the processes is that Auweter discloses the use of a water-miscible organic solvent, whereas Stein discloses the use of a water immiscible organic solvent in the first step of forming a suspension of a carotenoid. *Id.* Dr. Leunenberger expects that the processes of Stein and Auweter would produce carotenoid particle sizes in a comparable range of about 200 nm, which is much higher than the claimed range of

about 80 to about 120 nm. *Id.* Thus, Auweter and Stein provide no motivation for one skilled in the art to achieve the claimed powder composition. *Id.* at ¶ 27.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection of independent claim 29 over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested. Claims 30, 31, 33-35 various depend from claim 29, and are also not rendered obvious.

**17. Claim 30 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 30 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 29, wherein the crude emulsion formed has a solids content of from about 30%(wt) to about 50%(wt)." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 30 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**18. Claim 31 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 31 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 29, further comprising mixing the crude emulsion prior to emulsifying, to provide a crude emulsion having droplets which are about 1500 nm or less." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 31 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**19. Claim 33 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 33 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 29, wherein the emulsifying occurs at a pressure of from about 20,000 psi (about 1,360 bar) to about 35,000 psi (about 2380 bar)." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 33 obvious.

Also, as disclosed above, Dr. Leunenberger describes in his Declaration that Auweter discloses a process that differs from the process used in making the claimed powder composition, and does not provide any guidance as to how the presently claimed composition can be produced. Declaration, ¶ 28.

Moreover, Auweter's disclosure of the use of atmospheric or superatmospheric pressure, e.g., in the range of 20 to 80 bar, preferably 30 to 60 bar, to produce a cold-water dispersible powder, provides no indication, or expectation of success, of using a high-pressure homogenization process at a pressure of from about about 20,000 psi (about 1,360 bar) to about 35,000 psi (about 2380 bar), as disclosed. *Id.* and Auweter, Col. 2, ll. 59-62. The range pressure indicated for Auweter is much less than the range indicated for the instant invention, and application of the instantly claimed pressure range would have been a matter beyond routine experimentation.

Also, Dr. Leunenberger's Declaration explains that even if one were to consider using greater pressure, although Auweter provides no suggestion of this, the literature indicates that pressure is not a result-effective variable in relation to the diameter of particles in an emulsion. *See* Declaration, ¶ 29.

As for Stein, Dr. Leuenberger states that the process of Stein is similar to the process of Auweter in achieving their mutual goal of preparing carotenoid powder compositions for use as colorants. *Id.* at ¶ 30. The principle difference between the processes is that Auweter discloses the use of a water-miscible organic solvent, whereas Stein discloses the use of a water immiscible organic solvent in the first step of forming a suspension of a carotenoid. *Id.* Dr. Leuenberger expects that the processes of Stein and Auweter would produce carotenoid particle sizes in a comparable range of about 200 nm, which is much higher than the claimed range of about 80 to about 120 nm. *Id.* Thus, Auweter and Stein provide no motivation for one skilled in the art to achieve the claimed powder composition. *Id.* at ¶ 27.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

## **20. Claim 34 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 34 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 29, wherein the emulsifying occurs at a pressure of about 25,000 psi (about 1,700 bar)." The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 34 obvious.

The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 29 obvious.

Also, as disclosed above, Dr. Leunenberger states in his Declaration that Auweter discloses a process that differs from the process used in making the claimed powder composition, and does not provide any guidance as to how the presently claimed composition can be produced. Declaration, ¶ 28.

Moreover, Auweter's disclosure of the use of atmospheric or superatmospheric pressure, e.g., in the range of 20 to 80 bar, preferably 30 to 60 bar, to produce a cold-water dispersible powder, provides no indication, or expectation of success, of using a high-pressure homogenization process at a pressure of about 25,000 psi (about 1,700 bar), as disclosed. *Id.* and Auweter, Col. 2, ll. 59-62. The range pressure indicated for Auweter is much less than the range indicated for the instant invention, and application of the instantly claimed pressure range would have been a matter beyond routine experimentation.

Also, Dr. Leuenberger's Declaration explains that even if one were to consider using greater pressure, although Auweter provides no suggestion of this, the literature indicates that pressure is not a result-effective variable in relation to the diameter of particles in an emulsion. *See Declaration*, ¶ 29.

As for Stein, Dr. Leuenberger states that the process of Stein is similar to the process of Auweter in achieving their mutual goal of preparing carotenoid powder compositions for use as colorants. *Id.* at ¶ 30. The principle difference between the processes is that Auweter discloses the use of a water-miscible organic solvent, whereas Stein discloses the use of a water immiscible organic solvent in the first step of forming a suspension of a carotenoid. *Id.* Dr. Leuenberger expects that the processes of Stein and Auweter would produce carotenoid particle sizes in a comparable range of about 200 nm, which is much higher than the claimed range of about 80 to about 120 nm. *Id.* Thus, Auweter and Stein provide no motivation for one skilled in the art to achieve the claimed powder composition. *Id.* at ¶ 27.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

## **21. Claim 35 is Independently Patentable**

For at least the reasons set forth above in Section VII, Appellants respectfully disagree with the Examiner's rejection over claim 35 and submit that this claim is separately patentable. The cited reference fails to provide a teaching or suggestion of "[t]he powder composition according to claim 29, wherein the emulsion-forming composition is an oil-in-water

emulsion-forming composition.” The Examiner has failed to provide any evidence that the compositions or methods described in Auweter in view of Lorant or Auweter and Stein in view of Lorant render claim 35 obvious.

For at least the above, Appellants respectfully submit that the 35 USC 103(a) rejection over Auweter in view of Lorant or Auweter and Stein in view of Lorant is improper and withdrawal of the rejection is respectfully requested.

**B. Claim 36 is not obvious over either Auweter in view of Lorant and Cannalunga or Auweter and Stein in view of Lorant and Cannalunga**

The Examiner asserts that Auweter, Stein, and Lorant fails to teach the claimed moisture content, however, Cannolunga teaches vitamin powder preparations comprising emulsifying fat soluble vitamins with water, gelatin and gum acacia and a sugar or a sugar alcohol, wherein the compositions are dry and free-flowing. Office Action dated June 22, 2011, page 7. The Examiner asserts that the example teaches maintaining moisture content to less than 8%, thus, it would have been obvious for one of ordinary skill in the art to prepare the powders of Auweter in nanoparticle ranges suggested by Stein, in the size ranges as low as 30-200 nm of Lorant and further maintain the moisture content of the composition to less than 8% and even at 3% because Cannalunga suggests that the low moisture content prevents agglomeration of the particles dispersed. *Id.* at pages 7-8.

As set forth above, however, it is not obvious for one of ordinary skill to combine the teachings of Auweter, Stein, and Lorant to obtain the instant claimed powder composition, and Howard does not overcome these deficiencies. Therefore, the Examiner has failed to set forth a *prima facie* case of obviousness.

### **VIII. Conclusion**

Appellants respectfully submit that the Examiner has not set forth a proper *prima facie* case of obviousness because no reasoning has been articulated based on rational underpinnings to support the legal conclusion of obviousness. Withdrawal of the rejections are respectfully requested.

The requisite fee due upon filing of this brief is submitted herewith. Any additional fee is to be charged to Baker Donelson Bearman Caldwell & Berkowitz, PC, Deposit Account No. 50-4254, referencing docket number 2921268-001000.

Respectfully submitted,

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## **IX. Claims Appendix**

1-2. (Canceled)

3. (Previously Presented) The powder composition according to claim 36, wherein the polysaccharide gum is isolated from the group consisting of plants, animals and microbial sources.

4. (Previously Presented) The powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of exudate gums, seaweed gums, seed gums, microbial gums, and mixtures thereof.

5. (Previously Presented) The powder composition according to claim 3 wherein the polysaccharide gum is selected from the group consisting of gum arabic, flaxseed gum, ghatti gum, tamarind gum, arabinogalactan, and mixtures.

6. (Previously Presented) The powder composition according to claim 5 wherein the polysaccharide gum is gum arabic.

7. (Previously Presented) The powder composition according to claim 36 wherein the protein is isolated from a plant or an animal source.

8. (Previously Presented) The powder composition according to claim 7 wherein the protein is selected from the group consisting of sunflower proteins, soy-bean proteins, cotton seed proteins, peanut proteins, rape seed proteins, milk proteins, blood proteins, egg proteins, gelatine, crosslinked gelatine, and mixtures thereof.

9. (Previously Presented) The powder composition according to claim 8 wherein the protein is gelatine.

10. (Previously Presented) The powder composition according to claim 36 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E or its esters, vitamin A or its esters, vitamin K (phytomenadione), vitamin D3 (cholecalciferol), and mixtures thereof.

11. (Previously Presented) The powder composition according to claim 10 wherein the fat-soluble vitamin is selected from the group consisting of vitamin E acetate, vitamin A acetate, vitamin A palmitate, and mixtures thereof.



12. (Previously Presented) The powder composition according to claim 36 wherein the ratio of fat-soluble vitamin to matrix component is from about 1:99 to about 3:1.
13. (Previously Presented) The powder composition according to claim 12 wherein the ratio of fat-soluble vitamin to matrix component is from about 1:8 to about 1:1.
14. (Previously Presented) The powder composition according to claim 36 wherein the composition comprises from about 60 to 85% by weight of a matrix component, based on the total weight of all the components present in the composition.
17. (Previously Presented) The powder composition according to claim 36, which is formed into a tablet.
28. (Previously Presented) The powder composition according to claim 36 wherein the emulsion-forming composition is an oil-in water emulsion-forming composition.
29. (Previously Presented) A powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets, wherein the powder composition is produced by a process comprising:
- (a) combining water with a matrix component selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, to form a solution;
  - (b) adding a fat-soluble vitamin to the solution to form a crude emulsion, wherein the fat-soluble vitamin is added in an amount to provide from about 10% to about 30% by weight fat-soluble vitamin in the powder composition;
  - (c) emulsifying the crude emulsion at a temperature of from about 5° C to about 75° C at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar), to obtain an emulsion in which the droplets have an average diameter of about 80 to about 120 nm; and

- (d) drying the emulsion to obtain the powder composition.
30. (Previously Presented) The powder composition according to claim 29, wherein the crude emulsion formed has a solids content of from about 30%(wt) to about 50%(wt).
31. (Previously Presented) The powder composition according to claim 29, further comprising mixing the crude emulsion prior to emulsifying, to provide a crude emulsion having droplets which are about 1500 nm or less.
33. (Previously Presented) The powder composition according to claim 29, wherein the emulsifying occurs at a pressure of from about 20,000 psi (about 1,360 bar) to about 35,000 psi (about 2380 bar).
34. (Previously Presented) The powder composition according to claim 29, wherein the emulsifying occurs at a pressure of about 25,000 psi (about 1,700 bar).
35. (Previously Presented) The powder composition according to claim 29 wherein the emulsion-forming composition is an oil-in water emulsion-forming composition.
36. (Previously Presented) A powder composition consisting of at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets having an average diameter of about 80 to about 120 nanometers (nm) and wherein the fat-soluble vitamin is present in the powder composition in the amount of from about 10% to about 30% by weight and wherein the composition has a moisture content of about 1 to 4% by weight.

## **X. Evidence Appendix**

1. Declaration of Dr. Bruno Leuenberger, dated November 12, 2009, originally submitted with RCE dated December 7, 2009

a. Exhibit A: Dr. Bruno Leuenberger's CV

b. Exhibit B: Pozharski *et al.*, "Relationship between Turbidity of Lipid Vesicle Suspensions and Particle Size," *Analytical Biochemistry* (2001) 291,158-162.

c. Exhibit C: A copy of the Stein Declaration

d. Exhibit D: The Merck Index, Fourteenth Ed. (2006) 1853, 1854, and 9494.

e. Exhibit E: Desmumaux *et al.*, "Formation of Sunflower Oil Emulsions Stabilized by Whey Proteins with High-Pressure Homogenization (up to 350 Mpa); Effect of Pressure on Emulsion Characteristics," *Intl. J. Food Science and Tech.* (2002), 37, 263-269.

f. Exhibit F: Robin, *et al.* "Microfluidization of dairy model emulsions. I. Preparation of emulsions and influence of processing and formulation on the size distribution of milk fat globules," *Lait* (1992) 72, 511-531.

2. Declaration of Dr. Hermann Stein, dated March 16, 2005, originally submitted with response dated March 28, 2005.

**XI. Related Proceedings Appendix**

NONE



Docket No.: 20523 US (C038435/0120240)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Chyi-Cheng CHEN and Bruno LEUENBERGER

Serial No.: 09/726,880

Filed: November 30, 2000

For: **A VITAMIN POWDER COMPOSITION  
AND METHOD OF MAKING**

Examiner: L. Channavajjala

Art Unit: 1611

**DECLARATION OF BRUNO LEUENBERGER, Ph.D., UNDER 37 C.F.R § 1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

I, Bruno Leuenberger, a Swiss citizen, declare as follows:

1. I received a Ph.D in Physical Chemistry from the University of Bern, Switzerland, in 1985. My postdoctoral work was done at the University of Virginia, Charlottesville, VA, USA, in 1986.

2. I have been employed by DSM Nutritional Products Ltd., Kaiseraugst, Switzerland ("DSM") or its predecessor, Roche Vitamins Division, Basel, Switzerland ("Roche Vitamins"), since 1987. DSM became my employer during or around December 2003.

3. Specifically, from 1987 to 1989, while working at Roche Vitamins, I was a Laboratory Leader, Product Formulation. During 1990, I was the Group Head of

Product Form Development Research. From 1991 to 2001, I was the Section Head of Carotenoid Formulation and the Project Manager of certain carotenoid-related projects. From 2001 to 2004, I was the Section Head of Product Form Development, Human Nutrition & Health and I was the Project Manager of several formulation projects. From 2004 to 2007, I was the Competence Manager, Product Form Concept & Design. From 2007 to the present, I have been a Corporate Scientist, Nutrition Formulation and Application. My work overall has involved development of product forms of carotenoids, vitamins and other health-related ingredients for feed, food, and pharmaceuticals, with emphasis on formulation of sparingly soluble actives and controlled release. I have also investigated raw materials, especially protective colloids, for the development of new product forms. In addition, I have evaluated new formulation technologies, especially pertaining to micronization, emulsification and solidification. A copy of my Curriculum Vitae is enclosed as Exhibit A.

4. The present application discloses and claims a powder composition comprising at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets having an average diameter of about 80 to about 120 nanometers (nm) and wherein the fat-soluble vitamin is present in the powder composition in the amount of from about 10% to about 30% by weight. The fat-soluble vitamin may be, for example, vitamin E or its esters, vitamin A or its esters, vitamin K (phytomenadione), vitamin D<sub>3</sub> (cholecalciferol), and mixtures thereof. The specification

discloses that the fat-soluble vitamin "may be used in pure form, or in a suitable diluent such as a fat or edible oil (e.g. soybean oil). Thus the droplets in the powder of this invention may contain one or more vitamins in an appropriate diluent." (Page 10, lines 5-7.)

5. The specification also discloses that "[t]he invention contemplates powder compositions which, when added to a liquid, provide vitamin droplets averaging about 70 to 200 nm in diameter, ... preferably about 80 to about 120 nm.... When the liquid is optically clear, then the liquid remains for all practical purposes optically clear after addition of a powder composition according to this invention." (Page 6, lines 19-24.) The specification also discloses that "[t]he compositions of this invention may ... be added to liquids that are not optically clear, [yet] increase in turbidity is not of concern. Ringing will not occur." (Page 6, lines 17-19.)

6. The specification further discloses that "[o]ptical clarity (turbidity) can be measured by a turbidimeter." (Page 5, lines 27-28.) Furthermore, "[t]urbidity is measured by directing a beam of light into a cell containing a test sample, measuring the amount of light that is reflected at a 90 degree angle by any droplets present in the sample and comparing it to the light scattered by a standard reference suspension. ... The amount of reflected light is directly proportional to the degree of turbidity. NTU (Nephelometric Turbidity Unit) is customarily used to describe the results from the turbidity measurement using a turbidimeter. Higher NTU means higher turbidity." (Page 6, lines 3-11.) The specification further discloses that "optical clarity (NTU) is a function of the droplet size of the composition." (Page 23, lines 4-5.) This is indicated, for example, in Figure 2, which "shows a graph of droplet size (nm) versus optical clarity

(NTU) for 15.75%(wt) vitamin E (circle) and 26.25%(wt) vitamin E (square) in water dispersion." (Page 4, lines 9-10; Fig. 2.) As can be seen in the graph, the optical clarity (NTU) of a claimed powder composition which is in a water dispersion is lower, i.e., improved, for emulsion droplet particles between about 80 to about 120 nanometers (nm), than for emulsion droplet particles above that particle size range. (Fig. 2.)

7. As noted above, the application discloses and claims that the matrix in which the fat-soluble vitamin is dispersed consists of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof. The specification discloses that "[t]he polysaccharide gum and/or the protein, as used in this invention both have sufficient emulsifying properties. This means that they have sufficient emulsifying properties in an oil-in-water context to emulsify the oil into a fine dispersion in an aqueous medium and are capable of forming a stable emulsion of a desired droplet size ... under conditions of high pressure homogenization." (Page 6, lines 26-31.) Whether a selected polysaccharide gum or a protein has an emulsifying capacity sufficient for use in the claimed invention may be easily determined by assaying whether or not the selected polysaccharide gum and/or protein can maintain an emulsion as defined above, and as further described..." (Page 7, lines 7-11.) The protocol that one skilled in the art can use to determine whether or not a potential matrix component has a sufficient emulsifying capacity and is thus suitable for use in accordance with the claimed invention can be found in the specification at page 7, lines 12-23. Further information regarding the matrix component can be found in the



specification from page 6, line 31 to page 7, line 6, and page 7, line 26 to page 9, line 28.

8. In addition, the present application discloses and claims a powder composition comprising at least one fat-soluble vitamin dispersed in a matrix of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets, wherein the powder composition is produced by a process comprising: (a) combining water with a matrix component selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, to form a solution; (b) adding a fat-soluble vitamin to the solution to form a crude emulsion, wherein the fat-soluble vitamin is added in an amount to provide from about 10% to about 30% by weight fat-soluble vitamin in the powder composition; (c) emulsifying the crude emulsion at a temperature of from about 5° C to about 75° C at a pressure of from about 10,000 psi (about 680 bar) to about 60,000 psi (about 4080 bar), to obtain an emulsion in which the droplets have an average diameter of about 70 to about 200 nm; and (d) drying the emulsion to obtain the powder composition. The specification discloses that "[i]n order to attain the desired droplet size, the emulsion step ... may be repeated through one or more passes as necessary to obtain the desired droplet size, i.e., the crude emulsion is passed into the homogenization vessel, emulsified, passed out of the homogenization vessel, and passed through the homogenization vessel again until the desired droplet size is attained." (Page 21, lines 12-16.) The specification provides guidance in

disclosing that "[u]sually at least five to twenty passes will be required." (Page 21, line 16.) It is further disclosed that "[e]mulsification passes should continue until testing shows that the desired droplet size is achieved as determined by particle size analysis (for example, by light scattering...). It is important that the homogenization step be performed at an ultra-high pressure as described ... to effectively reduce the droplet size of the emulsion to a desirable size." (Page 21, lines 23-29.) As disclosed, "homogenization" refers to "further emulsification." (Page 26, lines 4-5.) The specification also discloses that "the droplet size of the emulsion determines the droplet size in the resulting powder..." (Page 22, lines 8-11, and page 23, lines 25-30.) Emulsification may be continued, for example, until the droplets in the emulsion have a preferred average diameter of from about 80 to about 120 nm. (Page 21, line 27 to Page 22, line 7.)

9. I am aware that an Office Action issued on April 1, 2009, with regard to the present application. (Paper No. 20090330.) It is my understanding that in the Office Action, the Examiner asserted that claims 1, 3-14, and 17 are not patentably distinct from U.S. Patent No. 5,968,251 to Auweter et al. ("Auweter") alone, or in view of EP 937412 to Stein et al. ("Stein"). (Id. at 2.) The Examiner has relied on these documents as disclosing and/or suggesting a powder composition comprising at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets having an average diameter of about 80 to about 120 nanometers (nm) and

wherein the fat-soluble vitamin is present in the powder composition in the amount of from about 10% to about 30% by weight. (Id. at 2-3.)

10. It is also my understanding that in the Office Action, the Examiner asserted that claim 15 is not patentably distinct from Auweter alone or in view of Stein and U.S. Patent No. 3,886,294 to Emodi et al. ("Emodi"). The Examiner has relied on these documents as disclosing and/or suggesting a powder composition as indicated in paragraph 9 above, wherein the composition further comprises from about 60 to 85% by weight of a matrix component, based on the total weight of all the components present in the composition, and the composition has a moisture content of about 1-4% by weight.

11. I am familiar with Auweter cited in the pending Office Action. Auweter discloses that carotenoids are "entirely insoluble" in water. (Col. 1, lines 42-51.) Auweter set out to prepare coloring compositions using carotenoids, and sought to overcome the "poor coloring results" that had previously been obtained due to the insolubility of carotenoids. (Id.)

12. Auweter further discloses "coldwater-dispersible dry powders which contain carotenoids and are obtainable [by the process disclosed] and which have different color effects depending on the production variant." (Col. 1, lines 26-29.) Auweter also discloses that the "[c]arotenoid preparations in the form of coldwater-dispersible powders are produced by

- a) preparing a molecular-disperse solution of a carotenoid, with or without an emulsifier and/or an edible oil, in a volatile, water-miscible, organic solvent at elevated temperature and adding therein an aqueous solution of a protective colloid, whereupon the hydrophilic solvent component is transferred into the aqueous phase, and

- the hydrophobic phase of the carotenoid results as [the] nanodisperse phase,
- b) heating the resulting hydrosol at from 40° C. to 90° C., with or without cooling of the hydrosol to from 0° C. to 30° C. beforehand, and
  - c) removing the solvent and the water from the heated hydrosol, and converting it into a water-dispersible dry powder. (Abstract.)

Auweter discloses that the molecular disperse solution according to process step a) can be carried out "under atmospheric or superatmospheric pressure." (Col. 2, lines 47-54.) According to Auweter, "[s]uperatmospheric pressure, e.g. in the range from 20 bar to 80 bar, preferably 30 to 60 bar, may be advantageous for rapid preparation of the molecular-disperse solution." (Col. 2, lines 59-62.) Auweter also discloses that in example 1, "[t]he ... process took place under 30 bar..." (Col. 6, line 48-49.)

13. "Examples of protective colloids which are used" in accordance with Auweter are said to be "gelatin, fish gelatin, starch, dextrin, vegetable proteins, pectin, gum arabic, casein, caseinate or mixtures thereof." (Col. 4, lines 40-42.) Auweter discloses that "it is also possible to employ polyvinyl alcohol, polyvinylpyrrolidone, methylcellulose, carboxymethylcellulose, hydroxypropylcellulose and alginates." (Col. 4, lines 43-46.)

14. Auweter also discloses that "[t]he result is a dry powder which, on use of a water-soluble colloid, can be dissolved again in water to obtain a uniform dispersion of the active substance with particle sizes in the range below 1  $\mu\text{m}$ . The active substance hydrosol obtained in this way proves to be extremely stable..., despite the fine particles." (Col. 5, lines 28-34.) Auweter further discloses that "[t]he preparations according to the invention are, by reason of their good coldwater dispersibility, outstandingly suitable as coloring agents ..." (Col. 5, lines 35-37.) In

addition, Auweter discloses that one of the process variants results in "the precipitated active substance particles after heating [being] essentially spherical with a diameter of, typically, 200 nm." (Col. 3, lines 52-57.) There is no disclosure in Auweter of any particle size of precipitated active substance that does not have at least a 200 nm diameter in one direction.

15. Auweter further discloses that Example 1, which includes  $\beta$ -carotene, dl- $\alpha$ -tocopherol, and gelatin, "[resulted] in a colloid-disperse  $\beta$ -carotene dispersion with a yellow hue. Spray drying the dispersion resulted in a free-flowing dry powder which forms a clear yellow dispersion in water." (Col. 6, lines 47-48 and 52-58.) In Example 2, it is disclosed that " $\beta$ -[c]arotene was precipitated in colloid-disperse form as described in Example 1." (Col. 6, lines 61-62.) Auweter further discloses that upon processing, Example 2 "[resulted] in a colloid-disperse  $\beta$ -carotene dispersion with an orange hue. Spray drying of the dispersion resulted in a free-flowing dry powder which forms a clear orange dispersion in water." (Col. 6, line 61 to Col. 7, line 2.)

16. I am also familiar with Stein cited in the pending Office Action. Stein discloses "a continuous process for the preparation of a pulverous carotenoid, retinoid or natural colourant preparation, wherein the active ingredient is finely divided ...." (Abstract). The process includes the steps of:

- a) forming a suspension of the active ingredient in a water-immiscible organic solvent optionally containing an antioxidant and/or an oil,
- b) feeding the suspension of step a) to a heat exchanger and heating said suspension to 100-250°C, whereby the residence time in the heat exchanger is less than 5 sec,
- c) rapidly mixing the solution of step b) at a temperature in the range of 20-100°C with an aqueous solution of a swellable colloid optionally containing a stabilizer,
- d) removing the organic solvent and

e) converting the dispersion of step d) into a pulverous preparation. (Col. 2, lines 3-16.)

The "finely divided" starting material is said to be of "a particle size of less than 1.5 micron, preferably less than 1 micron, more preferably less than 0.4 micron." (Col. 2, lines 18-21.) Stein further discloses that the "swellable colloid" can include gelatin, carbohydrates, dextrin, pectin, gum arabic, octenylbutanedioate amylopectin, milk proteins, and vegetable protein, or mixtures thereof. (Col. 3, lines 2-8.) Stein also discloses that powders formed from the compositions are soluble in cold water and provide coloration. (See Examples 1-5.)

17. I am also familiar with the Declaration of Mr. Hermann Stein Under 37 C.F.R. § 1.132 dated March 16, 2005 ("the Stein Declaration"), which is of record. I understand that the Stein Declaration was provided to the U.S. Patent and Trademark Office with the Submission Under 37 C.F.R. § 1.114; Response to Final Office Action on March 24, 2005 (which was received in the U.S. Patent and Trademark Office on March 28, 2005). It is noted that the Declarant of the Stein Declaration, Mr. Hermann Stein, is the same Hermann Stein who is an inventor of the Stein document cited by the Examiner. A copy of the Stein Declaration is attached as Exhibit C.

18. I am also familiar with Emodi cited in the pending Office Action. Emodi discloses "[l]iquid and powder carotenoid coloring compositions suitable for the preparation of optically clear, stable aqueous solutions, and the preparation thereof..." (Abstract, lines 1-3.) Emodi also discloses that "[t]he composition [consists] of one or more carotenoid coloring substances, an antioxidant and an emulsifier ingredient comprising a polysorbate and a mixture of monoglycerides of low molecular weight saturated coconut fatty acids and up to two optional emulsifier components selected

from the group consisting of a mixture of saturated fractions of coconut oil triglycerides and a polyoxyethylene (40) stearate wax." (Abstract, lines 3-11.) Emodi further discloses that "[t]he carotenoid coloring compositions, ... whether in the liquid or powder form, contain from about 0.1 to about 2.0 percent by weight, preferably from about 0.3 to about 1.0 percent by weight of the carotenoid coloring agent." (Col. 2, lines 13-17). Each of the powder composition examples, Examples 1, 2, and 4-7, includes either 0.3 percent, 0.5 percent, or 1 percent of a carotenoid compound. (Col. 4, line 50 to Col. 5, line 39, and Col. 6, line 1 to Col. 7, line 41.) The powder form of Emodi's compositions is also said to include "a water-soluble carrier composition which comprises a sugar, e.g., sucrose, fructose, lactose, invert sugar and the like and a water-soluble colloid-former such as, for example, hydrolyzed gelatin, low or high bloom gelatin and mixtures of hydrolyzed cereal solids and sugar..." (Col. 2, lines 25-33.) In addition, Emodi discloses that "aqueous solutions of the compositions ... formed from the various liquids and powders can be passed through a filter which will retain particles larger than 0.22 micron without loss of color." (Col. 4, lines 30-34.)

19. Emodi also discloses that "the powdered carotenoid coloring compositions of the invention are prepared by initially forming a supersaturated carotenoid liquid [which is] prepared by heating the combined components of the emulsifier ingredient and the preservative and dissolving the carotenoid coloring agent therein. A temperature of from about 80° to about 140°C. preferably from about 100° to about 120°C. is contemplated." (Col. 4, lines 6-9 and Col. 3, line 63 to Col. 4, line 1.) Emodi further discloses that the supersaturated carotenoid liquid "while still at the formation temperature, i.e. preferably at a temperature of from about 100° to 120°C., [is

added] to a previously formed aqueous solution containing the carrier composition, i.e. the soluble colloid-forming component, sugar and preservatives. The combined solutions are thereafter spray dried utilizing conventional spray drying equipment. The resultant spray dried powder is composed of submicroscopic droplets of a solution of a carotenoid coloring material in emulsifier ingredient encased in a water soluble colloidal film." (Col. 4, lines 9-19.) Example 1 of Emodi is said to provide a powder preparation containing 0.5 percent by weight  $\beta$ -apo-8'-carotenal. The spray dried powder is disclosed as "having a moisture content of less than 2 percent by weight." (Col. 4, line 50 to Col. 5, line 12.)

20. As noted above, the claimed powder composition achieves optical clarity. (See, e.g., ¶¶ 5-6 above.) The specification describes that there is a direct relationship between the amount of light reflected from any droplets present and the degree of turbidity which is reported in units of NTU (Nephelometric Turbidity Unit). (See ¶ 6 above.) As also noted above, the specification discloses that optical clarity (NTU) is a function of the droplet size of the claimed composition, as shown in Figure 2. (See ¶ 6 above.)

21. The general relationship between turbidity of lipid vesicle suspensions and particle size is indicated, for example, in Pozharski *et al.*, Relationship Between Turbidity of Lipid Vesicle Suspensions and Particle Size, (2001) 291, 158-162 (received September 15, 2000) ("Pozharski"). (Exhibit B.) As can be seen in Figure 1 on page 159, turbidity is very low for particles having smaller diameters. For particle diameters below 200 nm, turbidity approaches zero. Pozharski discloses that the "size-



turbidity dependence [data, including data not shown, indicates] that such behavior is inherent in the properties of lipid vesicles as light scatters." (Page 159, lines 2-7.)

22. Furthermore, at a particle size of about 200 nm, particles are at a transitional point between the invisible and visible ranges of the spectrum. At decreasing particle sizes below 200 nm, particles enter a size range which is not visible to the eye. Particles having an average diameter in a range of from about 80 to about 120 nm are in the invisible size range. Based on the foregoing, I believe that these particles are small enough to allow sufficient transmission of light such that a clear liquid in which a powder composition having such particles is added appears as a transparent and/or translucent solution which is essentially free from turbidity. Thus, the claimed average diameter particle size range of about 80 to about 120 nm of the fat soluble vitamin in the form of solid droplets is integral to the favorable aspects achieved with the claimed powder composition of optical clarity and a transparent and/or translucent solution upon addition to a clear liquid.

23. The physicochemical properties of carotenes such as  $\beta$ -carotene and fat-soluble vitamins such as  $\alpha$ -tocopherol, for example, are known to differ markedly. See, e.g., selected portions of The Merck Index, Fourteenth Ed. 2006 ("The Merck Index"), attached as Exhibit D. As indicated in the entry for  $\beta$ -carotene in The Merck Index,  $\beta$ -carotene is "[p]ractically [insoluble] in water", and its melting point is 183°C. (Exhibit D, page 1854, lines 15 and 19.) Thus,  $\beta$ -carotene is an insoluble solid compound. The use indicated is as a "[y]ellow coloring agent for foods." (Exhibit D, page 1854, line 26.) The melting point of  $\alpha$ -tocopherol, on the other hand, is 2.5-3.5° C. (Exhibit D, page 9494, under the structure of  $\alpha$ -tocopherol.) Thus,  $\alpha$ -tocopherol is a

liquid oil, and it is known to be light or amber yellow in color. The physicochemical properties of  $\beta$ -carotene and fat-soluble vitamins differ in significant ways that affect the relative goals in formulating. In looking to formulate a liquid oil to achieve a powder composition having optical clarity, i.e., being essentially free from turbidity in solution, and resulting in a transparent and/or translucent solution in a clear liquid, one skilled in the art would not have looked to technologies for formulating a solid, insoluble, colored compound which are for preparing compositions having color effects.

24. In view of my extensive knowledge in the field, it is my understanding that given the stated goal of each of Auweter, Stein, and Emodi of providing powder coloring compositions, colorant particles in the disclosed formulations of these documents would have an average diameter range such that the particles would be visible to the eye.

25. From my review of Auweter, it is my understanding that Auweter provides a  $\beta$ -carotene powder composition that renders  $\beta$ -carotene, which is otherwise insoluble, dispersible as fine particles in cold water. It is also my understanding that the dispersion disclosed by Auweter is a solid in liquid dispersion stabilized with colloids. In view of the foregoing and Auweter's disclosed particle size of about 200 nm, it is my understanding that the coldwater-dispersible powder composition, when added to a previously clear liquid, would produce a turbid and colored solution. This is consistent with Auweter's stated goal of a coldwater-dispersible powder composition that provides color effects.

26. It is my opinion that the coldwater-dispersible composition of Auweter is significantly different from the presently claimed powder composition.

Auweter's  $\beta$ -carotene powder composition provides fine particles of  $\beta$ -carotene which would disperse as solid particles in liquid, whereas the claimed powder composition provides solid droplets of a fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof. The claimed emulsion-forming composition has sufficient emulsifying properties in an oil-in-water context to emulsify the oil into a fine dispersion in an aqueous medium and forms a stable emulsion of the claimed droplet size. Whereas Auweter's  $\beta$ -carotene powder composition having particle sizes of about 200 nm would produce a turbid and colored solution, the claimed powder composition having solid droplets of fat soluble vitamin with an average diameter of about 80 to about 120 nm achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid.

27. Based on the foregoing, Auweter provides no motivation for one skilled in the art to achieve the claimed powder composition. In fact, the use of  $\beta$ -carotene in Auweter to provide color effects is antithetical to the claimed powder composition which achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid. Auweter's disclosure of "precipitated active substance particles ... with a diameter of, typically, 200 nm", a particle size which would appear visible in an otherwise clear solution, also flies in the face of obtaining the claimed powder composition which comprises solid droplets of a fat-soluble vitamin having an average diameter of about 80 to about 120 nm, which droplets are not in the visible range.

28. Auweter does not disclose a process using an emulsion-forming composition that has sufficient emulsifying properties in an oil in water context to form a stable emulsion of the claimed droplet size. Auweter's process differs from the process used in making the claimed powder composition. Auweter discloses how to make a solid in liquid dispersion of fine particles of  $\beta$ -carotene stabilized with colloids. Furthermore, Auweter provides no guidance as to how the presently claimed composition could be produced. For example, Auweter's disclosure of the use of atmospheric or superatmospheric pressure, e.g., in the range of 20 to 80 bar, preferably 30 to 60 bar, to produce a cold-water dispersible powder, provides no indication to use a high-pressure homogenization process, as disclosed.

29. Even if one were to consider using greater pressure, although Auweter provides no suggestion of this, the literature indicates that pressure is not a result-effective variable in relation to the diameter of particles in an emulsion. For example, Desrumaux and Marcand investigated the effect of pressure on the emulsification of sunflower oil (20%) in water using an ultra-high-pressure homogenizer. Desrumaux, A. and Marcand, J., Formation of Sunflower Oil Emulsions Stabilized by Whey Proteins with High-Pressure Homogenization (up to 350 MPa); Effect of Pressure on Emulsion Characteristics, *Intl J. Food Science and Tech.* (2002) 37, 263-269 ("Desrumaux"). (See Exhibit E.) Desrumaux discloses:

Homogenization reduced the Sauter diameter appreciably, the reduction increasing with treatment pressure from 50 to 90 MPa (Fig. 4). This result agrees with the study of Robin *et al.* (1992), who observed a decrease in the droplet average size between 7.8 and 76.3 MPa. Above 90 MPa, the [droplet diameter] increased with pressure (Fig. 4) and then stabilized approaching 200 MPa. Robin *et al.* observed a similar plateau of the droplet size diameter, but between

60 and 76.3 MPa.... Above 200 MPa, the [droplet diameter] decreased and then increased again at around 250 MPa. However, there was a final decrease of [droplet diameter] above about 300 MPa." (Page 267.)

Robin *et al.*, Microfluidization of Dairy Model Emulsions. I. Preparation of Emulsions and Influence of Processing on the Size Distribution of Milk Fat Globules (1992) *Lait*, 72 511-550 ("Robin"), which is cited by Desrumaux, is also attached as Exhibit F. Thus, the literature indicates that particle diameter has no consistent relation to pressure.

30. It is my understanding that the process of Stein is similar to the process of Auweter in achieving their mutual goal of preparing carotenoid powder compositions for use as colorants. The principle difference between the processes is that Auweter discloses the use of a water-miscible organic solvent, whereas Stein discloses the use of a water immiscible organic solvent in the first step of forming a suspension of a carotenoid. I am not aware of any reason why the very similar processes of Stein and Auweter would not achieve substantially the same results in terms of carotenoid particle size. Moreover, I would expect that the processes of Stein and Auweter would produce carotenoid particle sizes in a comparable range.

31. In the Stein Declaration, Mr. Hermann Stein declares that "[d]uring the research that led to the invention disclosed in Stein, my co-inventors and I, using the knowledge available at the time, attempted to produce the smallest possible particle size." (Paragraphs 6, lines 1-3.) Stein reproduced Example 5 of Stein, "with a view toward optimizing the process by producing the smallest particle size." (Paragraph 6, lines 3-4.) Example 5, which is found at Col. 6, paragraphs 54-58 of Stein, is also reprinted in paragraph 6 of the Stein Declaration.

32. Stein declares that "[a]s Example 5 shows, at that time, at best we could produce particle sizes of about 196 nm. (Paragraph 7, lines 1-2.) Stein also declares that "[b]ased on my unique knowledge of the methods and compositions of Stein, and my long experience in the area of the production carbohydrate matrices, it is my opinion that one of skill in the art at the time of the above-captioned invention familiar with the disclosure of Stein could not have produced particles of the presently claimed size." (Paragraph 7, lines 2-6.) Stein further declares that "it is also my opinion that one could not have predicted that the process of the present invention would produce significantly smaller particle sizes than the methods of Stein." (Paragraph 8.)

33. I agree with H. Stein's analysis and conclusions. Accordingly, in view of all of the foregoing, it is my opinion that one skilled in this art could not have predicted from Auweter alone or in view of Stein that the claimed powder composition could be produced having a fat soluble vitamin in the form of solid droplets having an average diameter of about 80 to about 120 nm, and which achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid.

34. Emodi's disclosure of powder carotenoid coloring compositions having only about 0.1 to about 2.0 percent by weight, preferably from about 0.3 to about 1.0 percent by weight of a carotenoid coloring agent, in addition to the required emulsifiers, does not suggest the claimed powder composition having a fat-soluble vitamin in the amount from 10% to about 30% by weight of the composition. In view of Emodi, one skilled in the art would have been led toward low percentages of active substance in combination with emulsifiers, rather than to the claimed powder composition. Also, it is my understanding that Emodi's disclosure that various liquids

and powders can be passed through a filter which retains particles larger than 0.22 micron without loss of color indicates that Emodi's powder contains particles larger than 0.22 micron, and that colored particles retained in the filtered solution have a particle size in the visible range.

35. In view of the foregoing, it is further my opinion that one skilled in the art could not have predicted from Auweter alone or in view of Stein and Emodi that the claimed powder composition could be produced having a fat soluble vitamin in the form of solid droplets having an average diameter of about 80 to about 120 nm which is present in the powder composition in the amount of from about 10% to about 30% by weight, and having from about 60 to 85% by weight of a matrix component based on the total weight of all the components present in the composition, wherein the composition has a moisture content of about 1-4% by weight, and achieves optical clarity and a transparent and/or translucent solution upon addition to a clear liquid.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12. Nov. 09

  
Bruno Leuenberger

## Short Curriculum Vitae of Dr. Bruno H. Leuenberger

**Work Field:** Development of product forms of carotenoids, vitamins and health ingredients for the industry segments feed, food and pharma; with special emphasize on formulation of sparingly soluble actives and controlled release.  
Search for novel raw materials, especially protective colloids, for the development of new product forms.  
Evaluation of new formulation techniques and technologies, especially in the field of micronization, emulsification and solidification.  
Collaboration with Marketing and Production for existing and new product forms.  
Collaboration and network building with external partners and universities in the field of formulation and application.

06/07 - today Corporate Scientist Nutrition Formulation and Application

10/04 – 05/07 Competence Manager Product Form Concept & Design

06/01 - 09/04 Section Head of Product Form Development Human Nutrition & Health.  
Project Manager of several formulation projects.

01/91 - 05/01 Section Head of Carotenoid Formulation.  
Project Manager of selected carotenoid projects.  
Promotions: 2000 Scientific Expert  
1998 Power of attorney (Prokura)  
1994 Power to act (Handlungsvollmacht)

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receptor kinase. Orthovanadate activates insulin receptor kinase by inhibiting the activity of a protein tyrosine phosphatase in the cells. Cephalochromin is a polyphenolic compound that has been observed to activate the IRTK activity (unpublished observation). Compound A is a small molecule that has been shown to enhance IRTK in these cells (21, 22). As shown in Fig. 2C, insulin receptor isolated from cells that have been treated with these agents showed a higher IRTK activity than the untreated cells. Moreover, this activity was similar to what has been observed for these compounds using the radioactive version of IRTK assay (date not shown). Taken together, these data demonstrate the usefulness of this assay for measuring the kinase activity of the insulin receptor that has been treated with diverse kinds of activators.

In summary, we have presented a significant improvement in the method to measure insulin receptor tyrosine kinase activity. This has been accomplished by developing a FRET-based assay, which utilizes a biotinylated substrate peptide from a natural substrate site of the IRTK, a Eu-labeled pT66, an anti-phosphotyrosine antibody that specifically recognizes the phosphorylated peptide, and XL665-labeled streptavidin. Utilizing these reagents, we have demonstrated that this detection method yields results similar to what would be expected with the radioactive version of this type of assay. We have also shown that this assay is not only able to detect insulin-stimulated activation of the receptor kinase activity, but is also able to measure increases in the kinase activity of the insulin receptor that has been stimulated with compounds known to enhance insulin receptor kinase activity in cells. In contrast to other formats this assay is performed in a single plate without various washing steps which are often necessary to remove unincorporated ATP from the reaction. Due to this feature of the assay we have been able to process more than twenty-five 96-well plates per day. In short, this assay is a very robust and efficient method of determining the status of insulin receptor kinase isolated from cells *in vitro* without the use of radioactive materials.

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## Relationship between Turbidity of Lipid Vesicle Suspensions and Particle Size

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Various physical methods exist for determining the distribution of sizes of micron and submicron particles in suspension. Light and electron microscopy, viscosimetry, sedimentation techniques, the electrical sens-

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ing zone method (Coulter technique), various scattering techniques (static and dynamic light scattering, X-ray and neutron diffraction), chromatography, electrophoresis, and some other more exotic methods have been used for sizing. In liposome applications, the most common in recent years has been the dynamic light scattering technique. The method is relatively fast and easily applied to normal samples without special preparation. Also, it can be extended to assess the shape and size distributions. Electron microscopy, although more complicated, invasive, and time-consuming, helps to obtain detailed and reliable information about lipid particles having an extended size range or non-spherical shape.

A rapid and reliable method of estimating particle size is especially useful when dealing with lipid vesicles and, despite the numerous methods mentioned above, none of them combines both speed and ready availability of the required experimental equipment. In this paper we describe a method for estimating an average size of lipid vesicles with only a spectrophotometer. Theoretical considerations and experimental evidence allow us to propose two possible measurement schemes, both based on calibration with vesicles of known size: (i) fit turbidities measured with more than one vesicle sample of known size to a standard curve; (ii) obtain the turbidity spectrum for a vesicle sample of known size and convert it to the size dependence of turbidity. The vesicle samples of known size can readily be produced by extrusion (1, 2). The details of both approaches are discussed further.

#### Experimental Approach (Procedure 1)

Figure 1 represents the experimentally obtained relationship between the turbidity of a lipid suspension and the size of the constituent vesicles. Egg L- $\alpha$ -phosphatidylcholine (Avanti) at 1 mg/ml was used, samples of different sizes having been obtained by sonication for different durations of time in a water bath sonicator. The actual diameter of vesicles was measured by dynamic light scattering (Brookhaven Instruments BI-200SM goniometer and BI-9000 digital correlator; correlation curves analyzed by quadratic cumulants (3)). The turbidity was measured as the apparent optical density with a Beckman DU-50 spectrophotometer at 440 nm (upper curve) and 780 nm (lower curve).

The first important observation is the general shape of the curve. The turbidity as a function of vesicle diameter fits two intersecting lines, being linear for small and constant for large sizes. These two lines intersect at some wavelength,  $\lambda_i$ , which is proportional to (although smaller than) the wavelength of the incident light. The plateau value of turbidity depends upon the wavelength of incident light. The turbidity at any wavelength of the sample of any particle size was pro-

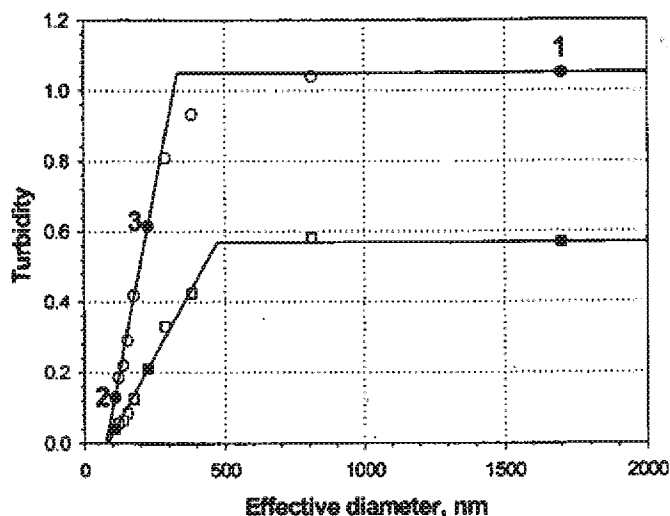


FIG. 1. General shape of the size-turbidity dependence for an egg L- $\alpha$ -phosphatidylcholine vesicle suspension at 1 mg/ml. Turbidity was measured at two wavelengths:  $\lambda = 440$  nm (circles) and 780 nm (squares). Closed symbols indicate size standards. Lines show the result of fitting by two intersecting lines as described in procedure 1.

portional to the lipid concentration up to 5 mg/ml (data not shown). We conducted similar experiments with egg lecithin and its 7:3 mixture (by weight) with cholesterol and in all cases obtained the same general size-turbidity dependence (data not shown), indicating that such behavior is inherent in the properties of lipid vesicles as light scatterers.

The explanation of the experimental size-turbidity dependence is given below in terms of Rayleigh-Debye scattering theory; however, even without a theoretical model, one can propose the following approach based only on the experimental results:

1. Measure the turbidity of a handshaken lipid suspension (in most cases these vesicles are larger than a wavelength, so it allows estimation of the turbidity maximum)—point 1 in Fig. 1.
2. Measure the turbidity of two standards smaller than the chosen wavelength (for instance, obtained by extrusion)—points 2 and 3 in Fig. 1.
3. Fit the above three values with two intersecting lines.

The linear region of the curve can then be used to assess the size of the unknown vesicles. The maximum turbidity, although useless by itself for size estimates, determines the range of sizes where a calibration is applicable.

#### Theory-Based Approach (Procedure 2)

The overall turbidity of a lipid vesicle suspension can be related to the scattering characteristics of the individual vesicle (4). If we consider a homogeneous ensem-

ble of thin shells as the model, one can show that turbidity,  $A$ , is given by<sup>2</sup>

$$A = 0.054 c S_0 N_A I \alpha_s / M \quad [1]$$

(see footnote for meaning of the symbols). The scattering efficiency,  $\alpha_s$ , is the fraction of light scattered by an individual vesicle of given size, and in order to complete the model it is necessary to apply scattering theory to obtain its analytical expression.

As long as a lipid vesicle is considered to be a thin shell or hollow sphere, the scattering efficiency can be determined within Rayleigh-Debye light scattering theory (5, 6). We generally followed this theory to derive an expression (below) for the scattering efficiency. Therefore, we will not present the complete derivation, which is rather straightforward, but limit our discussion to few important considerations.

*Rayleigh-Debye theory is applicable to lipid vesicles.* The applicability of Rayleigh-Debye theory is limited to the cases when there is no significant change of phase of the incident light when it penetrates the particle. In order to satisfy that condition, the thickness of the bilayer  $h$  (or stacked bilayers in multilamellar structure) should obey the condition,

$$h \ll \lambda_0 / (4\pi m_0(m-1)) \sim 1.1\lambda_0. \quad [2]$$

when  $m_0 \sim 1.33$ —refractive index of water;  $m = m_{\text{lipid}}/m_0 \sim 1.4/1.33 = 1.05$  and  $\lambda_0$  is the wavelength of incident light. Since the bilayer thickness is about 5 nm, even highly multilamellar structures obey the above condition, as long as visible or ultraviolet light is used. It is especially important to emphasize that it is

<sup>2</sup> The intensity of transmitted light,  $I$ , is given by

$$I = I_0 \exp(-\alpha_s n l),$$

where  $\alpha_s$  is scattering efficiency,  $n$  the concentration of particles,  $\sigma$  is their cross-sectional area,  $l$  the optical path length, and  $I_0$  the intensity of the incident light. Turbidity,  $A$ , is

$$A = -\log(I/I_0) = 0.43 \alpha_s n l.$$

The concentration,  $n$ , of unilamellar vesicles with bilayer thickness  $h$  and diameter  $D$  (assuming  $h \ll D$ ), is given by

$$n = c/m_0 = c S_0 N_A / 2\pi M D^2,$$

where  $c$  is the weight concentration of lipid,  $m_0$  the mass of individual vesicle,  $S_0$  the area per lipid molecule in the bilayer, and  $M$  the molecular weight of lipid. Taking into account that  $\sigma = \pi D^2/4$ , one can obtain

$$A = 0.43 \alpha_s (c S_0 N_A / 2\pi M D^2) (\pi D^2/4) l = 0.054 \alpha_s c S_0 N_A l / M.$$

the "longest dimension through the particle" (Ref. 7, p. 415), i.e., the bilayer thickness rather than its overall size (vesicle diameter), that is the decisive parameter for Rayleigh-Debye scattering. In the case of lipid vesicles, this means that it is still applicable when the diameter of the vesicle is comparable to or even larger than the wavelength.

*Lipid vesicle: Thin shell or hollow sphere?* The hollow sphere model of lipid vesicles is clearly closer to reality than that of a thin shell. However, this model leads to undesirable complications of the final equations. We compared the scattering efficiency  $\alpha_s$ , calculated for both models, and found that the correction introduced by the hollow sphere model is less than 0.2% in the case of unilamellar vesicles. Even for multilamellar vesicles with as many as 10 bilayers it is less than ~6%. We thus find Rayleigh-Debye scattering by thin shells to be adequate to describe the turbidity of lipid vesicle suspensions. The theory gives the following relationship between the turbidity  $A$  and diameter of vesicle  $D$

$$A = 4.82 \frac{c S_0 N_A I m_0^2 h^2 (m^2 - 1)^2}{M \lambda_0^2 (m^2 + 2)^2} \int_{\theta_0}^{\pi} \frac{\sin \theta (1 + \cos^2 \theta) \sin^2(2\pi m_0 D \sin(\theta/2)/\lambda_0)}{\sin^2(\theta/2)} d\theta. \quad [3]$$

where  $\theta_0$  is the acceptance angle of the detector (see footnote 2 for the meaning of other symbols). We introduce this parameter in order to indicate the fact that some part of the light scattered into small angles could impinge upon the detector, thus decreasing the apparent turbidity.

Calculations based on Eq. [3] show that, as size increases, turbidity rises to a plateau value, given a finite but small acceptance angle. The physical reason for this behavior is that larger particles scatter more and more light into the small scattering angle domain as diameter increases. As a consequence, experimental readings will strongly depend upon spectrophotometer geometry. We found, for instance, that the turbidity changes dramatically when the sample-detector distance is changed. The calibration procedure 1 (see above) and the experimental measurement must, therefore, be done under the same conditions (including sample concentration, lipid content, and measurement geometry). The problem of sample-detector geometry could be resolved by appropriate correction factors (8) or instrumentally, by using diaphragms (4), but doing so would considerably complicate the procedure.

Equation [3] can be rewritten in the following general form

$$A(D, \lambda) = f(D/\lambda)/\lambda^2. \quad [4]$$

The function  $f$  in the above equation can be obtained from the turbidity spectrum of a standard sample (known size) and then used to generate the size-turbidity relationship for a fixed wavelength. This consideration makes possible another mode of spectrophotometer calibration, utilizing only a single sample of known size.

1. Measure the turbidity spectrum (turbidity  $A$  as a function of wavelength) of a sample having a vesicle population of known size; i.e., obtain the function  $A_0(\lambda) = A(D_0, \lambda) = f(D_0/\lambda)/\lambda^2$ . As with procedure 1, the calibration sample can be produced by extrusion.

2. Calculate the size dependence of turbidity measured at some fixed wavelength  $\lambda_0$ , using the equation

$$\begin{aligned} A(D, \lambda_0) &= f(D/\lambda_0)/\lambda_0^2 = f(D_0/(\lambda_0 D_0/D))/\lambda_0^2 \\ &= f(D_0/\lambda^*)/\lambda_0^2 = \lambda^{*2} A_0(\lambda^*)/\lambda_0^2 \\ &= D_0^2 A_0(\lambda = \lambda_0 D_0/D)/D^2, \end{aligned} \quad [5]$$

where  $\lambda^* = \lambda_0 D_0/D$ .

It should be noted that this approach allows calibration only in a limited size range. Let us assume that a  $D_0 = 100$ -nm extruded sample is produced and the turbidity spectrum is obtained over the  $\lambda = 400$ – $800$  nm spectral range. Then, for  $\lambda_0 = 400$  nm, the calibration can be used for sizes  $D = \lambda_0 D_0/\lambda = 50$ – $100$  nm and, for  $\lambda_0 = 800$  nm,  $D = \lambda_0 D_0/\lambda = 100$ – $200$  nm. Naturally, this approach would benefit from any extension of spectral range.

### Concluding Remarks

Turbidity and turbidity spectra measurements have been successfully used in the past to estimate the average size of dispersed particles (see Ref. 7, pp. 325–343; 9, pp. 491–494). Interestingly, specific turbidity (i.e., turbidity normalized to concentration) for solid spheres at first increases with size, reaches a maximum, and then decreases for larger sizes. What accounts for the difference from the case of hollow spheres? If the scattering efficiency has a plateau because of interference of light scattered by different parts of the particle, this would seem to apply to solid spheres as well. Closer analysis reveals that a difference between shells and spheres is, in fact, expected. Equation [1] indicates that the only term which relates turbidity to size is the scattering efficiency of individual vesicles. In fact, turbidity is also proportional to the cross-sectional area of the particle (i.e., to  $D^2$ ) and to the number of particles in a unit volume, which for vesicles (i.e., thin shells) is inversely proportional to  $D^2$ . Since these two dependencies mutually cancel, nei-

ther appears in Eq. [1]. The situation is different if particles can be represented as a solid sphere. In that case their cross-sectional area is given by the same expression, but the particle concentration is inversely proportional to  $D^3$  (not  $D^2$ ), thus causing a decrease of the turbidity as a function of size, as long as the scattering efficiency increase is not so large as to overwhelm the particle concentration effect.

The range of sizes available for the method is limited by the wavelength of incident light, thus giving the maximal measurable size of about 500–600 nm (can be slightly extended if proper equipment is available to take readings in the infrared range). Theory predicts that one can extend this range by reducing the acceptance angle of the light detector; however, even with a zero aperture, the turbidity changes by only about 15% when the vesicle diameter drops from two to one wavelength. The accuracy of the procedure could thus decrease, even with precautions to minimize the acceptance angle.

Chong and Colbow (4) previously described a method to obtain vesicle sizes using the turbidity spectrum. Their method did not involve use of any standards and, as a consequence, required determination of the refractive index spectrum of the lipid. Determination of the refractive index is tedious and requires specialized equipment (10). On the other hand, the introduction of extrusion methods (1, 2) has made it very easy to prepare vesicle dispersions of known size. Turbidity-based vesicle size measurement has thus become eminently practical. Because the method presented here has important advantages of speed, simplicity, and common availability of the necessary equipment, it should be useful in laboratories in which liposome research is done.

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## Concurrent Measurement of Promoter Activity and Transfection Efficiency Using a New Reporter Vector Containing both *Photinus pyralis* and *Renilla reniformis* Luciferase Genes

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Reporter vectors are essential for the quantitative analysis of gene elements that potentially regulate mammalian gene expression (1). These gene elements may be *cis*-acting, such as promoters and enhancers, or *trans*-acting, such as various DNA-binding factors (2). Several kinds of reporter vectors have been developed to study the promoter and/or enhancer activities of genes: chloramphenicol acetyltransferase (CAT)<sup>2</sup> reporter vector,  $\beta$ -galactosidase reporter vector,  $\beta$ -glucuronase, alkaline phosphatase reporter vector, green fluorescent protein reporter vector, and luciferase reporter vector (3–7). Currently, reporter vectors using chemiluminescence-based assays ( $\beta$ -galactosidase reporter vector,  $\beta$ -glucuronase, alkaline phosphatase reporter vector, and luciferase reporter vector) are commonly used in many quantitative analyses of gene elements (8), because the sensitivity of the assays is

several orders of magnitude greater than that of conventional colorimetric- or fluorometric-based assays. Of them, luciferase reporter vectors are the most favored reporter vectors for functional analysis of promoters and enhancers of genes, due to their rapid, sensitive, and reproducible assay system (9). In the luciferase assay, luciferin and other components are added to cell extracts, and the production of light from both cell extracts expressing the luciferase gene is measured conveniently by a luminometer or scintillation counter (10).

Although luciferase reporter vectors have been commonly employed in numerous studies, the transfection of only luciferase vector cannot provide normalized values of the activities of gene elements without simultaneous transfection of a second reporter vector to measure the efficiency (11). In this paper, a novel reporter vector (pJDL<sub>cmv</sub>) was constructed to contain two luciferase genes, *Photinus pyralis* and *Renilla reniformis* luciferases, regulated by two different promoters for the first time, in order to measure simultaneously promoter activity and transfection efficiency. Two promoters of human glutaredoxin and ribonucleotide reductase R2 gene were investigated with this novel reporter vector to verify its appropriateness for simultaneous measurement of promoter activity and transfection efficiency.

## Materials and Methods

T4 DNA ligase and restriction enzymes were purchased from Promega (Madison, WI), Chameleon double-stranded site directed mutagenesis kit was from Stratagene (La Jolla, CA), and *Taq* polymerase and deoxynucleotides were from Perkin-Elmer (Norwalk, CT). Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) was used to determine the amount of protein.

**Cell culture conditions.** HeLa and NT cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were grown to 60% confluence for the transfection experiments.

**PCR cloning of *P. pyralis* and *R. reniformis* luciferase genes.** *P. pyralis* and *R. reniformis* luciferase genes were amplified from pGL and pRL vectors (Promega), respectively, by PCR using respective forward and backward primers corresponding to two genes: 5'-TGCT-TGGCATTCCGGTACTGTTGG-3' and 5'-TTTACAATT-TGGACTTTCCGCCCTTCTT-3' for the *Photinus* gene; 5'-CTGCAGAAGTTGGTCGTGAGGCAC-3' and 5'-TTGTTTCATTTTGTAGAACAGC-3' for the *Renilla* gene (12).

**PCR cloning of two DNA fragments containing poly(A) signal for *P. pyralis* and *R. reniformis* luciferase genes.** Two DNA fragments containing SV40 early and late poly(A) signals were respectively amplified from pEGFP (Clontech) and pRL (Promega) by PCR using forward and

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<sup>2</sup> Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; CMV, cytomegalovirus; poly(A), polyadenylation.

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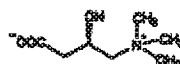
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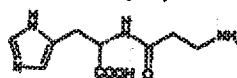
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Crystals from anhydrous ethanol + acetone, dec 196-198° (Carter, Bhattacharyya); also reported as crystals from isopropylalcohol, mp 200° (de) (Mauri). Very hygroscopic solid.  $[n]_D^{25}$  -21.3° ( $c = 10$  in water);  $[n]_D^{25}$  -37.8° ( $c = 10$  in water). Sol in water, hot alcohol. Practically insol in acetone, ether, benzene. Hydrochloride. [4645-48-1] Levocarnitine chloride; Flutidine.  $C_{15}H_{27}NO_2 \cdot HCl$ ; mol wt 317.46. Crystals, dec 142°.  $\alpha$ -Form. [406-74-8]  $\gamma$ -Amino- $\beta$ -hydroxybutyric acid ethyl ester;  $\gamma$ -trimethyl- $\beta$ -hydroxybutyrate. Synthesis: E. Sack *et al.*, *Ber. 86*, 525 (1953); H. E. Carter, P. K. Bhattacharyya, *J. Am. Chem. Soc.* 75, 2363 (1953). Hygroscopic crystalline solid, dec 193-197°. Sol in water, ethanol.  $\beta$ -Form hydrochloride. [463-85-2] Needles from ethanol, mp 196° (de). Very sol in water, not in hot ethanol; slightly sol in cold ethanol. Practically insol in acetone, ether.  $\alpha$ -Form. [341-14-0] Propyl. S. Friedman *et al.*, *Arch. Biochem. Biophys.* 66, 10 (1957). Crystals, dec 216-217°.  $[n]_D^{25} +30.9^\circ$ . Very sol in water and alcohol. Practically insol in acetone and ether.  $\beta$ -Form hydrochloride. [3007-44-4] Crystals, dec 142°. THERAP CAT: Vitamin (enzyme cofactor).

1852. Carosin. [705-84-0]  $\beta$ -Alanine- $\alpha$ -histidine; *ignotina*.  $C_9H_{12}N_2O_5$ ; mol wt 226.23. C 47.78%, H 4.24%, N 24.77%, O 31.23%. Naturally occurring dipeptide found in large amounts in skeletal muscle. Also present in other tissues such as brain, cardiac muscle, kidney. Water soluble antioxidant; functions as a free-radical scavenger. Isola: O. Witsch, *Amidolab. Ber.* 53, 1902 (1900); Wolff, Wilson, *J. Biol. Chem.* 95, 493 (1933); 169, 565 (1935). Synthesis from histidine and  $\beta$ -alanine or  $\beta$ -alanine-phenylalanine: Bauman, Ingvaldsen, *ibid.* 38, 271 (1918); Berg, Tulin, *Biochem. J.* 13, 406 (1918). Laser synthesis: S. Fried, de Vignaud, *J. Biol. Chem.* 108, 753 (1935); R. A. Turner, *J. Am. Chem. Soc.* 78, 2188 (1956); F. J. Vinick, S. Jung, *J. Org. Chem.* 46, 292 (1981). Crystal structure: H. Itoh *et al.*, *Acta Crystallogr.* 33B, 2959 (1977). Possible role in wound healing: D. E. Fisher *et al.*, *Proc. Soc. Exp. Biol. Med.* 158, 465 (1978). Review of physiological properties and therapeutic potential: S. E. Oshida, A. J. Sackin, *Adv. Aging* 28, 207-210 (2000).



Crystals from aqueous ethanol, mp 267° (de) (Vinick, Jung); also reported as mp 267° (capillary tube) and as mp 308-309° (Dennis *et al.*) (Lifshitz, de Vignaud).  $[n]_D^{25} +21.8^\circ$  ( $c = 1.5$  in water);  $pK_1$  2.66;  $pK_2$  6.21;  $pK_3$  9.51. Alkaline reaction. One gram dissolves in 1.1 ml water at 25°.  $\alpha$ -Form. [3452-88-3]  $C_{15}H_{27}N_2O_5$ . Crystals, dec 222°.  $[n]_D^{25} +24.1^\circ$  ( $c = 1.5$  in water). Very sol in water.  $\beta$ -Form hydrochloride. [3833-09-3]  $C_{15}H_{27}N_2O_5 \cdot HCl$ . Crystals, dec 142°. Very sol in water.

$\alpha$ -Form. [3833-09-3] Crystals, mp 260°.  $[n]_D^{25} -20.4^\circ$  ( $c = 1.5$ ).

1851. Caro's Acid. [7125-86-3] Peroxycarboxylic acid; sulfonoperoxide; peroxysulfonic acid.  $H_2O_2 \cdot S$ ; mol wt 114.08. H 1.77%, O 70.12%, S 28.11%. Dry reagent is prepared by stirring 10 g potassium persulfate into 11 g concentrated  $H_2SO_4$  for 10 min and adding 10 g finely powdered potassium sulfate; liquid reagent is obtained by stirring potassium persulfate with three times as much (by weight) of  $H_2SO_4$ ; all reagent is prepared by stirring 10 g potassium persulfate into 11 g concentrated  $H_2SO_4$  and adding 30 cc ice; Bazyar, Villiger, *Ber.* 33, 3623 (1899); another group by reacting 90%  $H_2O_2$  with chlorosulfonic acid at -40 to -50°. Ball, Edwards, *J. Am. Chem. Soc.* 78, 1123 (1956).

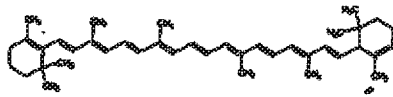


The product is a sticky liquid consisting of about equal amounts of Caro's acid and  $H_2SO_4$ .  $pK_a$  of Caro's acid 9.4  $\pm$  0.1. Oxygen is evolved at room temp; should be stored at dry ice temp.

**Caution:** Can be dangerously unstable, like most peroxides. Description of explosion at Brown University: J. O. Edwards, *Chem. Eng. News* 33, 3336 (1955). Explosion at Sun Oil, *ibid.* 28, 29 (Nov. 11, 1950). May be highly irritating to skin, eyes, mucous membranes.

**Use:** In prepn of dyes; oxidation of olefins to  $\alpha$ -glycols; oxidation of ketones to lactones or esters; treating woolens to prevent felting and shrinking; in bleaching compositions.

1852.  $\alpha$ -Carotene. [7488-99-3]  $C_{40}H_{56}$ ; mol wt 536.87. C 89.49%, H 10.51%. About as widely distributed as its  $\beta$ -isomer, but in smaller amounts. Best sources for both the  $\alpha$ - and  $\beta$ -isomers are carrots, palm-oil, and green leaves of various species. As a provitamin A it is half as active as  $\beta$ -carotene. Found in the mother liquor after crystallizing  $\beta$ -carotene, Isola by chromatography: Karrer, Walker, *Helv. Chim. Acta* 16, 641 (1933). Structures: Kuhn, Lederer, *Ber.* 64, 1349 (1931); Karrer *et al.*, *Helv. Chim. Acta* 14, 614 (1931); 16, 975 (1933). Natural (+)- $\alpha$ -carotene has 6'S configuration: Bagnat *et al.*, *ibid.* 53, 1729 (1949). Synthesis of di- $\alpha$ -carotene: Bagnat, Karrer, *ibid.* 38, 610 (1955); Tschamper *et al.*, *ibid.* 40, 1676 (1957); Bagg *et al.*, *ibid.* 44, 285 (1961).



Deep purple prisms, polyhedra from pure ether or from benzene + methanol, mp 187.50° (vacuumed tube).  $[n]_D^{25} +38.5^\circ$  ( $c = 0.08$  in benzene). Absorption max (CHCl<sub>3</sub>): 482, 434 nm. More sol than  $\beta$ -carotene. Freely sol in carbon disulfide, chloroform; sol in ether, benzene. Slightly sol in pure ether, alcohol. 189 mg benzene dissolves 294 mg at 0°. Practically insol in water, acids, alkalis. Absorbs oxygen from the air, giving rise to inactive colorless oxidation products. The oxidation is light in concentration. **Store in darkness in sealed ampoules and at low temp (-20°C).**

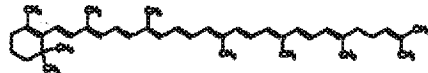
THERAP CAT: Vitamin A precursor.

THERAP CAT (VST): Vitamin A precursor for all species except can.

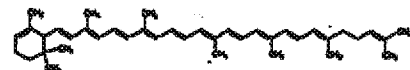
1853.  $\beta$ -Carotene. [7235-40-7]  $\beta$ -Carotene; Carotene; Provitamin; Solatene.  $C_{40}H_{56}$ ; mol wt 536.87. C 89.49%, H 10.51%. Most important of the provitamin A. Widely distributed in the plant and animal kingdom. In plants it occurs almost always together with chlorophyll. Isola from carrots: Wilbur, Zecher, *Z. Physiol. Chem.* 64, 47 (1910); Kuhn, Lederer, *Ber.* 64, 1349 (1931); Barnett *et al.*, US 3848308 (1958). Chromatography: Karrer, Walker, *Helv. Chim. Acta* 16, 641 (1933). Structure: Wilbur, Ming, *Ann.* 353, 1 (1907); Zechmeister *et al.*, *Ber.* 61, 566 (1928); 66, 123 (1933); Karrer *et al.*, *Helv. Chim. Acta* 13, 1142 (1929); 13, 1084 (1930); 14, 1033 (1931); Kuhn, Bruchmann, *Ber.* 63, 894 (1932); 66, 1319 (1933); 67, 1408 (1934); *Ann.* 516, 95 (1935). Crystal structure: Sterling, *Ann. Crystallogr.* 17, 1224 (1964). Synthesis: Dilaz *et al.*, *J. Am. Chem. Soc.* 72, 4844



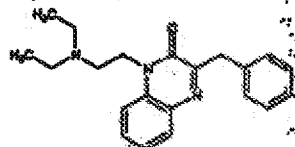
1834. *γ*-Carotene. [472-93-5].  $C_{40}H_{56}$ . Mol. wt. 536.8. C 89.49%, H 10.51%. A rare carotenoid. Has provitamin A activity. Best source is *Pantotheca schottiana*; Mass et al., *Arch. Biochem. Biophys.* 69, 150 (1957). Also present in small proportions in many plant products. It is a tetraterpene. It is a hydrocarbon, graphic isomer from *cis*-carotene. Kuhn, Brockmann, *Ber.* 64, 467 (1933); Winterstein, *Z. Physikal. Chem.* 219, 246 (1933). Structures: Kuhn, Brockmann, *loc. cit.*; *Neuzeitliche Vorfahren* 21, 44 (1933). Synthesis: Garbner et al., *Helv. Chim. Acta* 36, 1793 (1953); Rogers et al., *ibid.* 44, 925 (1961).



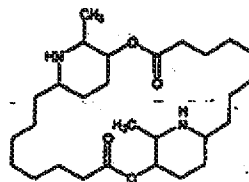
1855. *β*-Carotene, [472-92-4] *cis*-Carotene,  $C_{40}H_{56}$  mol wt 536.87, C 89.49%, H 10.51%. Extracted from the fruit of *Gonocarpus pruriens* Mig., *Fraxinacea*: Winterhagen, Z. Physiol. Chem. 219, 261 (1933). Occurs also in carrots and certain varieties of tomatoes. Isipis from tomato mutants: Porter, Murphy, Arch. Biochem. Biophys. 32, 21 (1951). Structure: Kargl, Quackenbush, ibid. 88, 59 (1960). Synthesis: Monodant et al., J. Chem. Soc. 1963, 2019. Absolute configuration: Buchecker, Eggen, Nativ. Chim. Acta, 37, 127 (1971).

CC12C=CC(C1)C(C2)C(C)C(C)O

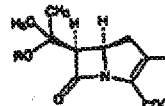
**1857.** Caroverine. [23465-76-1]  $\text{C}_{20}\text{H}_{29}\text{N}_3\text{O}_2$ .  $\text{C}_{20}\text{H}_{29}\text{N}_3\text{O}_2$ ; mol wt 365.47. C 72.38%, H 9.15%, O 8.47%. Prepn: Zellner *et al.*, US 3,822,000 (Dec. 1974). Jones Pharm.). Polarographic study: P. Pfäfel, *J. Pharm. Med.*, 167 (1969). Pharmacological study: F. Hahnke *et al.*, *Pharmazie-Zentralblatt*, 19 (1972).



1858. Carpaia. [3463-02-1]  $C_{11}H_{16}N_2O_2$ .  
70.23%, H 10.53%, N 8.55%, O 13.37%.  
Carpaia L. and in *Vasconcellea karwinskii* Carad. *Carpaia*  
*Carpaia* Karst. *Carpaia*, *Medicinal*, *off* L. *Carpaia*  
*Carpaia*, No. 7, 9 (1890); *Rapport*, *Baldridge*, J. *Am.*  
[1913]. *Reportedly* *can* *be* *used* *as* *brandy* *in* *CNS* *de-*  
*mentia*, *Arch. Int. Pharm.* 15, 84 (1906). *Structure*  
*of* *Carpaia* *et al.*, *Tamphodan* *Let.* 1963.  
*Carpaia*: *Colo. Rice*, J. *Org. Chem.* 30, 3463  
*Carpaia* *smiles*: *Carpaia*, J. *Indian Chem.*  
[1968].



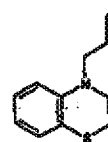
2859. *Corbellimyces*. Carbanoyl and olivanic acids, q.e. see below. Prodn of A and B by 199, now named *Simplicomyces olivaceus* (properties: NL 804) 1953 (1960, 1963 in Tokuda); A 7 (1960); by *Simplicomyces strain* 1356. Structure and abs config 1425 (1960); M. Nakayama et al. *syn study*: Y. Nozaki et al., *ibid.* 1359; A. N. Macgregor et al., *ibid.* 33, 403; M. Thara et al., *Hevra*, 1966; M. M. Raa, *J. Org. Chem.*, 1966; *Corbellomyces* A: T. Hino et al. *ibid.* 41; Aratani et al., *Tetrastich* et al., *ibid.* 2217.



Carpatomycin A. (76025-73-  
 Carpatomycin (Carpatomycin) 6-114  
 Carpatomycin 2.0 [hept-2-one-2-  
 Carpatomycin C-19393-H<sub>2</sub> antibiotic  
 Carpatomycin C<sub>2</sub>S<sub>2</sub> mol wt 342.37. Cod  
 Carpatomycin (S = 1.7 in water). uv max  
 Carpatomycin. (1980).

KA-6643-B; C-19393-S,  
series solid melting above  
100°C. w max (waxy): 240.2

1240. Carphenoxazine. [2-(4-(1-phenoxyl)propyl)-10-(4-(2-hydroxyethyl)-1-phenoxyl)-2-propoxyl-10-propylphenoxazine; Frek. 1240. C 67.73%, H 7.34%. J. M. Starck, N. Sperber. U.S. Pat. 3,223,146 (1966).



isolate. (2973-34-0)  
 Isolate from isopropanol.  
 Isolate from methanol, in  
 100% CAT: Andriyko  
 100% "

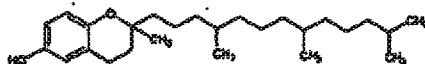
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mechanism of action: H. Pa, *Expert Opin. Ther. Targets* 8, 287-294 (2004).

THERAP CAT: Anti-inflammatory.

9494. Tocol. [119-38-2] 3,4-Dihydro-2-methyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-ol; 2-methyl-2-(4,8,12-trimethyltridecyl)-6-chromanol; 2-methyl-2-phytyl-6-chromanol; 6-hydroxy-2-methyl-2-phytylchromanol; 2-methyl-2-phytyl-6-hydroxychromanol.  $C_{31}H_{50}O_2$ ; mol wt 436.63. C 80.35%, H 11.41%, O 8.23%. Spontaneously by the condensation of hydroquinone and phytol in the presence of sulfur formic acid: Fendler, *Karrer, Helv. Chim. Acta* 40, 1837 (1957). Antioxidant activity of tocol and its methyl derivative: Otsu, van der Veen, *Lipids* 3, 331 (1968).

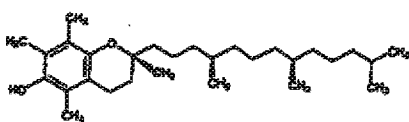


Colorless, viscous oil.  $b_p$  165-173°.

Acetate,  $C_{33}H_{54}O_2$ . Viscous oil.  $b_p$  180-185°.

USE: Antioxidant.

9495.  $\alpha$ -Tocopherol. [59-82-9] (2R)-3,4-Dihydro-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-6-chromanol;  $\alpha$ -tocopherol;  $\alpha$ -tocopherol; 5,7,8-trimethyl-2-methyl-2-phytyl-6-chromanol;  $\alpha$ -tocopherol.  $C_{55}H_{100}O_2$ ; mol wt 786.54. C 88.87%, H 11.70%, O 7.43%. Most bioactive of the naturally occurring forms of vitamin E, q.v. Richest sources are green vegetables, grains, and oils, particularly palm, safflower and sunflower oils. Isolated from wheat germ: H. M. Evans et al., *J. Biol. Chem.* 113, 319 (1936). Structures: R. Perle, *J. Am. Chem. Soc.* 83, 1154 (1961); 46, 700 (1924). Synthesis of  $\alpha$ -form: P. Karrer et al., *Helv. Chim. Acta* 33, 830, 832 (1950); F. Bergel et al., *J. Chem. Soc.* 1950, 1382. Distillation from vegetable oils and propyl acetate: J. G. Baxter et al., *J. Am. Chem. Soc.* 918 (1943). Preparation of crystalline natural form: C. D. Robinson, *ibid.* 1660. Preparation of crystalline acetate: *ibid.* 1687. Also study of natural  $\alpha$ -tocopherol: H. Mayr et al., *Helv. Chim. Acta* 46, 953 (1963). Stereoselective synthesis: K. K. Chen et al., *J. Org. Chem.* 43, 3433 (1978). Total synthesis of all 8 tocopherols: N. Cohen et al., *Helv. Chim. Acta* 64, 1158 (1981). Clinical trial in Alzheimer's disease: M. Sano et al., *N. Engl. J. Med.* 336, 1216 (1997). To improve immune function in healthy elderly: S. N. Meydani et al., *J. Am. Med. Assoc.* 277, 1380 (1997). Review of bioavailability from vitamin E supplements: M. G. Traber, *BioFactors* 18, 113-120 (1999). Review of clinical trials in heart disease: W. A. Pryor, *Free Radical Biol. Med.* 28, 141-164 (2000).



Transparent needles, mp 2.5-3.5°.  $[\alpha]_D^{25}$  -3.0° (benzene);  $[\alpha]_D^{25}$  +0.32° (ethanol).

Acetate, [59-85-7] Spondylvit.  $C_{57}H_{102}O_2$ ; mol wt 798.74. Light yellow oil. Crystallized at -30° as needle-like crystals, mp 26.3-27.3°.  $[\alpha]_D^{25}$  +0.32° ( $c = 10$  in chloroform);  $[\alpha]_D^{25}$  +0.32° (in ethanol).

Succinate, [4345-83-3]  $\alpha$ -Tocopheryl acid succinate; Tocovita. Needles from pear ether, mp 76-77°. uv max (ethanol): 286 nm ( $\epsilon_{286}^{1\%}$  38.5). Practically insol in water.

$\alpha$ -Tocopherol, [10191-41-0]  $\alpha$ -Tocopherol. Equimolar mixture of all four racemates. Slightly viscous, pale yellow oil.  $d_4^{20}$  0.950;  $b_p$  200-220°;  $n_D^{20}$  1.5043, uv max: 294 nm ( $\epsilon_{294}^{1\%}$  71). Practically insol in water. Freely sol in oils, fats, acetone, alcohol, chloroform, ether, other fat solvents. Stable to heat and alkalis in the absence of oxygen. Not affected by acids up to 100°. Slowly oxidized by atmospheric oxygen, rapidly by ferric and silver salts. Gradually darkens on exposure to light.

$\alpha$ -Tocopherol acetate, [52215-20-6]  $\alpha$ -Tocopheryl acetate; Dextolite; Ephyneol; Eucovin; Euton. Comprehensive descrip-

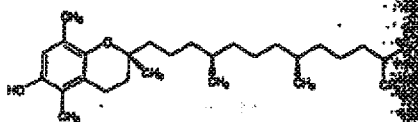
tion: S. C. Rudy, B. Z. Senkowski, *Anal. Profiles Drug Sub.* 111-126 (1974). Pale yellow, viscous liquid, mp -27.5°.  $d_4^{20}$  0.9533,  $b_p$  184°;  $b_p$  194°;  $b_p$  224°.  $n_D^{20}$  1.4930-1.4935, uv max (cyclohexane): 285.5 nm. Practically insol in water. Freely sol in acetone, chloroform, ether. Less readily sol in oils.

USE: As an antioxidant in vegetable oils and shortening.

THERAP CAT: Vitamin E supplement.

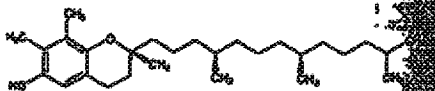
THERAP CAT (VET): Vitamin E supplement.

9496.  $\beta$ -Tocopherol. [14698-35-4]; [148-03-6] (2R)-3,4-Dihydro-2,5,8-trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,5,8-trimethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol; 5,8-dimethyl-2-methyl-2-phytyl-6-chromanol;  $\beta$ -tocopherol.  $C_{57}H_{100}O_2$ ; mol wt 786.54. C 88.87%, H 11.61%, O 7.68%. One of the naturally occurring forms of vitamin E, q.v. Is biologically less active than  $\alpha$ -tocopherol. May be separated by fractional crystallization: Emerson et al., *Science* 83, 421 (1936); *J. Biol. Chem.* 113, 319 (1936); Baxter et al., *J. Am. Chem. Soc.* 65, 918 (1943).



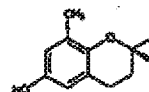
Pale yellow, viscous oil.  $b_p$  200-210°.  $[\alpha]_D^{25}$  +2.9° ( $c = 10$  in ethanol). uv max: 297 nm ( $\epsilon_{297}^{1\%}$  87.6). Insol in water. Freely sol in oils, fats, acetone, alcohol, chloroform, ether, other fat solvents. Very stable to heat and alkalis. Slowly oxidized by atmospheric oxygen, rapidly by ferric and silver salts. Gradually darkens on exposure to light.

9497.  $\gamma$ -Tocopherol. [34-28-4]; [7616-22-0] (2R)-3,4-Dihydro-2,7,8-trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol; (R,R,R)- $\gamma$ -tocopherol; 7,8-dimethyl-2-methyl-2-phytyl-6-chromanol;  $\gamma$ -tocopherol.  $C_{55}H_{100}O_2$ ; mol wt 786.54. C 88.87%, H 11.61%, O 7.48%. One of the naturally occurring forms of vitamin E, q.v. Most abundant tocopherol in soybean oil. Isolated by fractional crystallization: Emerson et al., *Science* 83, 421 (1936); *J. Biol. Chem.* 113, 319 (1936); J. G. Baxter et al., *J. Am. Chem. Soc.* 65, 918 (1943). Preparation of crystalline natural form: D. Robinson, *J. Am. Chem. Soc.* 65, 1660 (1943). Comparison of bioactivity with  $\alpha$ -tocopherol, q.v.: J. G. Blom, R. P. Evans, *ibid.* 164, 850 (1942). Protective effects vs reactive nitrogen species: R. V. Cooney et al., *Proc. Natl. Acad. Sci. USA* 94, 1093 (1997). E. Chabot et al., *ibid.* 94, 3217 (1997). HPLC determination: A. Sobczak et al., *J. Chromatogr. B* 730, 265 (1999). Review of bioavailability, metabolism, and activity: C. Traber et al., *Am. J. Clin. Nutr.* 74, 714-722 (2001).



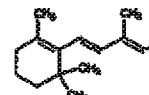
Pale yellow, viscous oil. Has been crystallized as trans needles, mp -3 to -2°.  $b_p$  200-210°.  $[\alpha]_D^{25}$  -2.4° ( $c = 10$  in benzene);  $[\alpha]_D^{25}$  +2.1° ( $c = 0.52$  in ethanol). uv max: 297 nm ( $\epsilon_{297}^{1\%}$  92.8). Insol in water. Freely sol in oils, fats, acetone, alcohol, chloroform, ether, other fat solvents. Very stable to heat and alkalis. Slowly oxidized by atmospheric oxygen, rapidly by ferric and silver salts. Gradually darkens on exposure to light.

9498.  $\delta$ -Tocopherol. [119-15-1] (2R)-3,4-Dihydro-2-methyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; 8-methyl-2-methyl-2-phytyl-6-chromanol.  $C_{57}H_{100}O_2$ ; mol wt 786.54. C 88.87%, H 11.52%, O 7.25%. One of the naturally occurring forms of vitamin E, q.v. Isolated from soybean oil: Stern et al., *J. Am. Chem. Soc.* 69, 899 (1947). Synthesis: Green et al., *J. Chem. Soc.* 1957, 2950-2955 (1957); Hoffmann-La Roche.



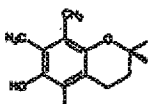
Pale yellow, viscous oil.  $b_p$  180-185° in benzene.

9499. Tocotrienol. 3,4-dihydro-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol;  $\alpha$ -tocotrienol.  $C_{88}H_{146}O_2$ ; mol wt 1378.34. C 88.87%, H 11.70%, O 7.43%. One of the naturally occurring forms of vitamin E, q.v. Richest sources are green vegetables, grains, and oils, particularly palm, safflower and sunflower oils. Isolated from wheat germ: H. M. Evans et al., *J. Biol. Chem.* 113, 319 (1936). Structures: R. Perle, *J. Am. Chem. Soc.* 83, 1154 (1961); 46, 700 (1924). Synthesis of  $\alpha$ -form: P. Karrer et al., *Helv. Chim. Acta* 33, 830, 832 (1950); F. Bergel et al., *J. Chem. Soc.* 1950, 1382. Distillation from vegetable oils and propyl acetate: J. G. Baxter et al., *J. Am. Chem. Soc.* 918 (1943). Preparation of crystalline natural form: C. D. Robinson, *ibid.* 1660. Preparation of crystalline acetate: *ibid.* 1687. Also study of natural  $\alpha$ -tocopherol: H. Mayr et al., *Helv. Chim. Acta* 46, 953 (1963). Stereoselective synthesis: K. K. Chen et al., *J. Org. Chem.* 43, 3433 (1978). Total synthesis of all 8 tocopherols: N. Cohen et al., *Helv. Chim. Acta* 64, 1158 (1981). Clinical trial in Alzheimer's disease: M. Sano et al., *N. Engl. J. Med.* 336, 1216 (1997). To improve immune function in healthy elderly: S. N. Meydani et al., *J. Am. Med. Assoc.* 277, 1380 (1997). Review of bioavailability from vitamin E supplements: M. G. Traber, *BioFactors* 18, 113-120 (1999). Review of clinical trials in heart disease: W. A. Pryor, *Free Radical Biol. Med.* 28, 141-164 (2000).



Light yellow oil. uv max (mg/kg): >100. THERAP CAT: Vitamin E.

9500.  $\alpha$ -Tocotrienol. (2R)-3,4-dihydro-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol;  $\alpha$ -tocotrienol.  $C_{88}H_{146}O_2$ ; mol wt 1378.34. C 88.87%, H 11.70%, O 7.43%. One of the naturally occurring forms of vitamin E, q.v. Richest sources are green vegetables, grains, and oils, particularly palm, safflower and sunflower oils. Isolated from wheat germ: H. M. Evans et al., *J. Biol. Chem.* 113, 319 (1936). Structures: R. Perle, *J. Am. Chem. Soc.* 83, 1154 (1961); 46, 700 (1924). Synthesis of  $\alpha$ -form: P. Karrer et al., *Helv. Chim. Acta* 33, 830, 832 (1950); F. Bergel et al., *J. Chem. Soc.* 1950, 1382. Distillation from vegetable oils and propyl acetate: J. G. Baxter et al., *J. Am. Chem. Soc.* 918 (1943). Preparation of crystalline natural form: C. D. Robinson, *ibid.* 1660. Preparation of crystalline acetate: *ibid.* 1687. Also study of natural  $\alpha$ -tocopherol: H. Mayr et al., *Helv. Chim. Acta* 46, 953 (1963). Stereoselective synthesis: K. K. Chen et al., *J. Org. Chem.* 43, 3433 (1978). Total synthesis of all 8 tocopherols: N. Cohen et al., *Helv. Chim. Acta* 64, 1158 (1981). Clinical trial in Alzheimer's disease: M. Sano et al., *N. Engl. J. Med.* 336, 1216 (1997). To improve immune function in healthy elderly: S. N. Meydani et al., *J. Am. Med. Assoc.* 277, 1380 (1997). Review of bioavailability from vitamin E supplements: M. G. Traber, *BioFactors* 18, 113-120 (1999). Review of clinical trials in heart disease: W. A. Pryor, *Free Radical Biol. Med.* 28, 141-164 (2000).



uv max (ethanol): 29.

9501.  $\beta$ -Tocotrienol. (2R)-3,4-dihydro-2,5,8-trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-2H-1-benzopyran-6-ol; (+)-2,5,8-trimethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol;  $\beta$ -tocotrienol.  $C_{88}H_{146}O_2$ ; mol wt 1378.34. C 88.87%, H 11.61%, O 7.68%. One of the naturally occurring forms of vitamin E, q.v. Isolated from soybean oil: Stern et al., *J. Am. Chem. Soc.* 69, 899 (1947). Synthesis: Green et al., *J. Chem. Soc.* 1957, 2950-2955 (1957); Hoffmann-La Roche.

## Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): effect of pressure on emulsion characteristics

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**Summary** A new ultra-high-pressure homogenizer was used to make very fine oil in water emulsions. The effect of pressures up to 350 MPa on sunflower oil (20%) in water emulsions was studied. The emulsifier used was whey protein concentrate (1.5%). The properties of the emulsions were characterized by laser light scattering (droplet size distribution) and coaxial cylinders rheometry (rheological behaviour). The protein adsorption fraction was obtained by a spectrophotometric method using bicinchoninic acid reagent.

Significant modifications in the structure and the texture of the emulsions were observed as the pressure increased. No change was revealed by polyacrylamide gel electrophoresis of the whey protein within the pressure range studied. Microdifferential scanning calorimetry scans indicated that the changes of the structural and textural properties may be because of changes in the protein conformation.

**Keywords** Droplet size, emulsification, microdifferential scanning calorimetry, protein adsorption, viscosity.

### Introduction

A large variety of foods are emulsions, from the more natural, e.g. milk, to the more sophisticated, e.g. sausages, mayonnaises. Emulsions are dispersions of liquid droplets in a liquid continuous phase. As the two liquids are immiscible, emulsions are very unstable. In order to stabilize emulsions, there must be surface active molecules at the interface of the droplet to prevent instantaneous coalescence.

Food emulsions are commonly produced in high-pressure homogenizers, in colloid mills or in batch reactors with high-speed blenders. Initially built for the homogenization of milk, high-pressure homogenizers are the most often used, as they give fine emulsions with precise texture properties (creams, ice creams) and higher degrees of stability. The principle of high-pressure homogenization is simple: a coarse emulsion produced with a

high-speed blender is forced under pressure through a narrow valve. The combination of the intense shear, cavitation and turbulent flow conditions in this valve leads to the disruption of fat globules (Walstra & Smulders, 1997; McClement, 1999). The decrease of the average size of the fat globules reduces the creaming velocity (Stokes law) and increases the stability of the emulsion. Food homogenizers usually go up to 60 MPa and the gap of the valves is typically between 15 and 300  $\mu\text{m}$ . Increasing the pressure and decreasing the gap size cause a greater degree of breakdown of droplets. Despite the large use of high-pressure homogenizers, few studies deal with the effect of very high pressures on the emulsions properties. Moreover, the studies are limited in the pressure range. According to Mulder & Walstra (1974) and Phipps (1975), the average fat globule diameter ( $d$ ) decreases with emulsification pressure ( $P$ ) in a relation  $d \propto P^{-0.6}$ , for pressures between 0.25 and 40.5 MPa. Davies (1985) described the breakdown of fat droplets with the Kolmogoroff theory and found a relation between the maximal droplet size

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and  $P^{-0.4}$ . According to Tornberg (1980), for pressures over 40 MPa and for a mass oil fraction of 12%, a phenomenon called 'overprocessing' occurs: the average droplet size increases with pressure. Robin *et al.* (1992) processed butter oil in water emulsions, with sodium caseinate as emulsifier, from 7.8 to 76.3 MPa in a microfluidizer. In the microfluidizer, two microstreams were projected under pressure against one another. They showed that the average size of fat globules decreased with pressure and reached a minimum around 60 MPa. Until now, no study has related the effect of 'dynamic' high pressures over 100 MPa on food emulsion formation. The effects of isostatic pressure on emulsions has been studied as a means of preservation. It has been shown that its influence on the already formed emulsions depends on the emulsifiers used (Dumay *et al.*, 1996).

Thus, the purpose of this work was to test the hypothesis that ultra-high-pressure homogenization (from about 50 to 350 MPa) significantly alters properties of oil-in-water emulsions when compared with more traditional pressure treatments.

## Material and methods

### Ingredients

Whey protein concentrate 'PS85' (85% protein), obtained by ultrafiltration of casein serum, was supplied by Eurial Poitou Touraine (Herbignac, France). The reported composition of this product is:  $N \times 6.38$ : 85%; fat: 3%; mineral salts: 4%; water content: 4%; pH  $6.5 \pm 0.2$ . The proteins are soluble over the complete pH range, and are good emulsifiers at pH < 7.

Sunflower oil was purchased from Lesieur (Neuilly-sur-Seine, France). Distilled water was filtered through a 0.2- $\mu$ m filter before use. Solutions of whey proteins (1.5%) were prepared using an ultraturrax rotor-stator system (Ika Labortechnik, Staufen, Germany) and stored at 4 °C.

### Ultra-high-pressure homogenizer

Coarse emulsions containing 20%, by mass, of sunflower oil and 80% of the whey protein concentrate aqueous solution were prepared at 4 °C, using an ultraturrax rotor-stator system and then passed through a homogenizer operating from 20 to

350 MPa (Stansted Fluid Power Ltd, Essex, UK). Emulsions were homogenized at different pressures in the range of 50–350 MPa. The homogenizing chamber was cooled with a cooling jacket containing cold water at 5 °C, in order to slow down the rise of temperature. Each emulsion was carefully collected and stored at 4 °C before analysing. This procedure was used to prevent any change in the size distribution of the fat globules because of churning. The experiments were duplicated.

The reproducibility of the high-pressure homogenization was tested by repeating an experimental point (20% oil, 150 MPa) four times.

### Light scattering measurements

The size distributions of the oil droplets were determined by the laser light scattering method. The diffractometer model used was the Mastersizer S (Malvern Instruments, Malvern, UK) equipped with a 300 reverse Fourier lens and a He-Ne laser ( $\lambda = 633$  nm). The emulsion was measured 5 min after ultra-high-pressure homogenization to cancel any creaming effect, and diluted to about 1/1000 with distilled water in the diffractometer cell, whilst stirring. Size distribution was presented as volume percentage vs. droplet diameter. The volume size distribution was calculated from the intensity of the light diffracted at each angle using Mie theory. The analysis requires a parameter known as the presentation value, a combination of the ratio of the relative refractive indices of the dispersed phase and water and the absorbance of the dispersed phase. The 3NAD presentation was used: oil (1.4564, 0.0000) in water (1.33), for which the absorbance value was selected after consultation with Malvern Instruments and verified using a carefully diluted emulsion of known concentration. The full size distribution was obtained using a polydisperse analysis, which allowed the calculation of the mean droplet diameter  $d_{32}$  (Sauter mean diameter) and a dispersion index called 'span', defined as

$$\text{span} = \frac{d[90] - d[10]}{d[50]}$$

where  $d[x]$  is the average droplet size in a volume in which  $[x]\%$  of the total sample weight remains constant. Measurements were repeated three times for each sample.

### Rheology measurements

Dynamic shear stress measurements were done at 20 °C with an AR 1000 Rheometer (TA Instruments, Waters Corporation, USA), equipped with a coaxial system (medium concentric cylinder with a conical end;  $R_1 = 13.83$  mm,  $R_2 = 15.0$  mm). Flow curves (shear stress vs. shear rate) were determined at increasing shear rates: 0–1200  $s^{-1}$  in 2 min (up and down flow curves).

### Protein surface concentration

Emulsion samples were centrifuged at 13 000 g for 30 min to separate the droplets from the aqueous serum phase. The supernatant (the cream) was carefully removed from the aqueous phase using a syringe. The cream layer was resuspended in ultrapure water to wash away any protein trapped between droplets, and the resulting emulsion was centrifuged again at 13 000 g for 30 min. The protein concentration of the two serums was determined by the Sigma Pierce spectrophotometric method using bicinchoninic acid reagent (BCA, Sigma procedure TPRO-562). This spectrophotometric method is based on the reduction of  $Cu^{2+}$  to  $Cu^+$  by the proteins (Biuret reaction).  $Cu^+$  reacts with the BCA and an intense purple complex is formed. This complex produces an absorbance maximum at 562 nm, which is directly proportional to the protein concentration (Smith *et al.*, 1985). A calibration curve was generated using bovine serum albumin (BSA) standard solution (Sigma, St Louis, USA), with a determination coefficient  $r^2 = 0.99$  (15 different concentrations used). The quantity of whey proteins absorbed was expressed in equivalent BSA and was calculated from the difference in the serum concentration prior to and after emulsification. The surface concentration of the absorbed protein was calculated from the known surface area per unit volume of emulsion and the difference in the amount of protein measured in the serum phase and the amount used to make the original emulsion, making allowance for any dilution.

### Electrophoresis (SDS-PAGE)

The protein fractions were determined by sodium dodecyl sulphate-polyacrylamide gel electrophor-

esis (SDS-PAGE), following the procedure described by Arrese *et al.* (1991). Gel slabs were fixed and stained simultaneously in aqueous solution containing 40% v/v ethanol, 7% v/v acetic acid and 0.025% coomassie brilliant blue R250. Proteins were denaturated with a treatment buffer containing 0.125 M Tris, 4% SDS v/v Glycerol, 0.2 M dithiotreitol, 0.02% bromophenol blue. About 50  $\mu g$  of protein was applied to each gel slot. Molecular weights of the protein bands were estimated by means of the SDS-70L kit (Sigma Chemical Co., St Louis, USA).

### Microdifferential scanning calorimetry

Microdifferential scanning calorimetry ( $\mu DSC$ ) was used to assess the degree of denaturation of the whey protein by the pressure of homogenization.  $\mu DSC$  thermographs of aqueous solutions of whey proteins before and after a single run in the homogenizer were prepared by using a Setaram 3 calorimeter (Caluire, France). Samples (700 mg) of 2% dispersions of whey proteins (pH 6.7) in distilled water were hermetically sealed in hastelloy C276 pans (volume = 1  $cm^3$ ). A closed pan filled with distilled water was used as the reference. The heating rate was fixed at 0.5 °C  $min^{-1}$  from 20 to 110 °C. Triplicate samples were analysed by  $\mu DSC$ .

### Results and discussion

#### Whey protein composition

Electrophoresis measurements indicated that the whey proteins were mainly composed of  $\beta$ -lactoglobulin (18 600 Da),  $\alpha$ -lactalbumin (14 200 Da), and serum albumin (66 000 Da) (Fig. 1). Examination of the  $\mu DSC$  curves showed three endothermic phenomena (Fig. 2): the first around 40–45 °C, the second between 60 and 65 °C and the third between 70 and 75 °C. These endothermic phenomena in whey proteins are explained by the behaviour of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, which are the predominant proteins (Paulsson *et al.*, 1985). The dimer form of  $\beta$ -lactoglobulin exists at  $5.2 < pH < 7.5$  as in our case (pH 6.7). Thus, according to McKenzie (1971) the first endothermic peak could be a dissociation of the dimers of  $\beta$ -lactoglobulin. The

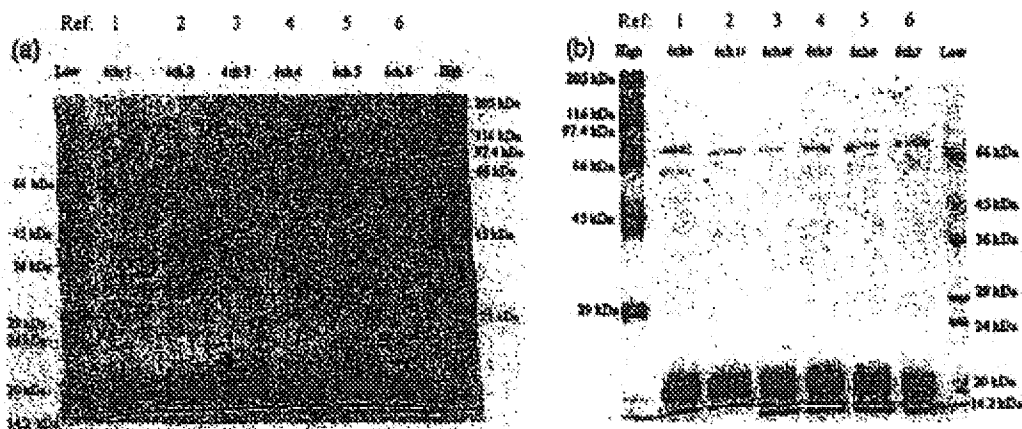


Figure 1 (a) SDS-PAGE of proteins before and after a single run in the homogenizer. Ref:  $\alpha$ -lactalbumin (14.2 kDa), trypsin (20 kDa), egg albumin (45 kDa), serum albumin (66 kDa). Column 1: before homogenization. Columns 2–6: after a single run in the homogenizer at 2: 30 MPa; 3: 60 MPa; 4: 90 MPa; 5: 120 MPa; 6: 150 MPa. (b) SDS-PAGE of protein after one run in the homogenizer. Ref: see (a). Column 1: 180 MPa; 2: 210 MPa; 3: 240 MPa; 4: 270 MPa; 5: 300 MPa; 6: 330 MPa.

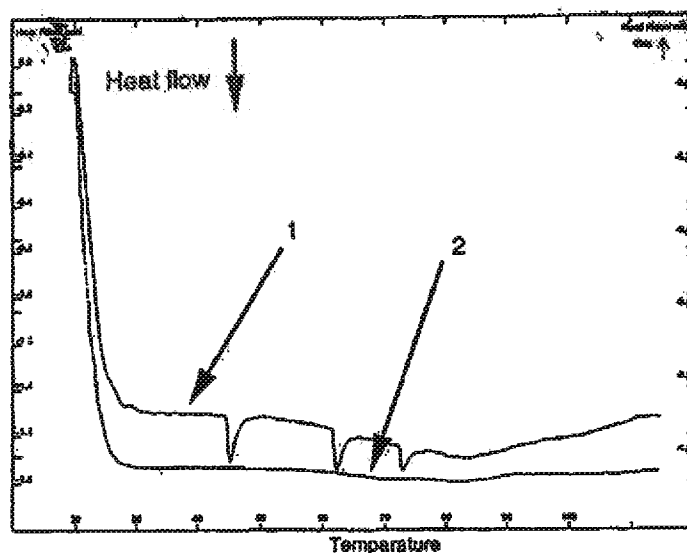


Figure 2 Micro-DSC graph of whey protein aqueous solutions (1.5%) before (1) and after (2) treatment at 300 MPa in the high-pressure homogenizer.

second endothermic peak at 60–65 °C corresponds at pH around 7 to the denaturation of the  $\alpha$ -lactalbumin, and the last endothermic peak around 70–75 °C corresponds to the denaturation of the  $\beta$ -lactoglobulin.

#### Reproducibility of the experiments

The reproducibility of the high-pressure homogenization after testing with an experimental point

(20% oil, 150 MPa) and repeated four times on four different days is shown in Table 1. According to the s.d. obtained, the reproducibility was satisfactory.

#### Evolution of temperature during the experiments

Despite the use of the cooling jacket, the temperature of the emulsion at the exit of the valve increased linearly with the pressure in the valve

Table 1 Reproducibility of the point 150 MPa, 20% oil

Temperature at the exit of the homogenizer	Sauter diameter $d_{32}$ ( $\mu\text{m}$ )	Size dispersion coefficient	Viscosity (Pa s)	Fraction of adsorbed proteins ( $\text{mg m}^{-2}$ )
40 °C (1)	0.68 (0.02)	3.75 (0.6)	0.009 (0.001)	0.77 (0.1)

Values within parentheses are standard deviations.

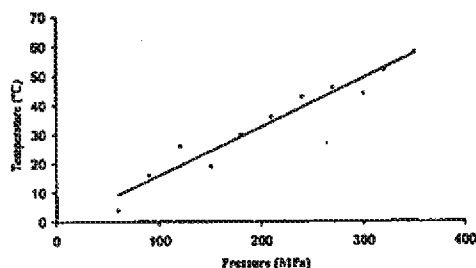


Figure 3 Effect of pressure on the temperature at the exit of the homogenizer.

(Fig. 3). Above 300 MPa, the temperature of the emulsion at the exit is close to the temperatures of the peaks observed by  $\mu\text{DSC}$ .

#### Effect of high pressure on emulsion properties

The Sauter diameter before homogenization was around 30  $\mu\text{m}$ . Homogenization reduced the Sauter diameter appreciably, the reduction increasing with treatment pressure from 50 to 90 MPa (Fig. 4). This result agrees with the study of Robin *et al.* (1992), who observed a decrease in the droplet average size between 7.8 and 76.3 MPa. Above 90 MPa,  $d_{32}$  increased with pressure (Fig. 4) and then stabilized approaching 200 MPa. Robin *et al.* (1992) observed a similar plateau of the droplet size diameter, but between 60 and 76.3 MPa. This phenomenon can be referred to as 'overprocessing,' as stated by Tornberg (1980): the average droplet size is stable over a certain range of pressure, and increases at higher pressures. The density of energy (up to  $10^{12} \text{ W m}^{-3}$ ) is estimated by knowing the flow rate and the pressure drop inside the valve, and is partially dissipated as heat (Fig. 3), which can lead to an increase of the Sauter diameter: the Brownian motion increases and so also the probability of collision and coalescence.

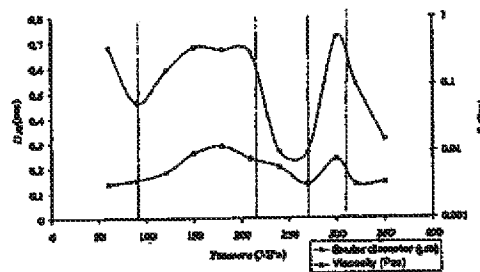


Figure 4 Effect of the pressure of homogenization on the Sauter diameter  $d_{32}$  and the viscosity  $\eta$  of the emulsions.

Above 200 MPa,  $d_{32}$  decreased and then increased again at around 250 MPa. However, there was a final decrease of  $d_{32}$  above about 300 MPa. This final decrease could be explained by the increased probability of rupture. At this level of pressure, the shear rate inside the valve is enormous, the probability of rupture again becomes higher than the probability of coalescence and  $d_{32}$  decreases.

The effects of high-pressure homogenization on the sizes dispersion coefficient are interesting: above 90 MPa, as the pressure increased, the dispersion coefficient strongly decreased (seven-fold) (Fig. 5). Also, the droplet size distribution was bimodal for the lower pressures, but at higher

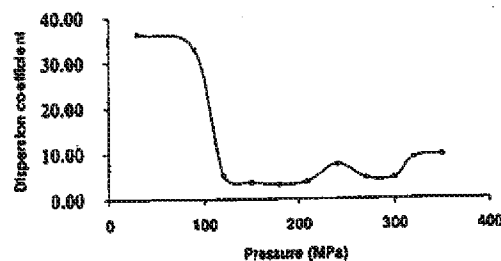


Figure 5 Effect of pressure of homogenization on the size dispersion coefficient of the droplet.

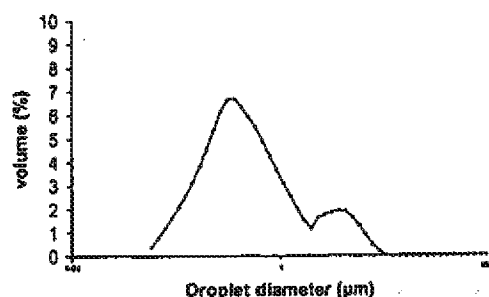


Figure 6 Size distribution of the oil droplet of the emulsion after a single run in the homogenizer at 300 MPa.

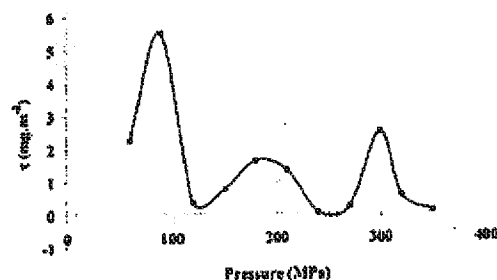


Figure 7 Effect of pressure of homogenization on the fraction of adsorbed proteins.

pressures the second peak decreased (Fig. 6). Thus, the coalescence rate of the droplets is reduced because a large dispersion favours rapid coalescence. Above 90 MPa, the main effect of high-pressure homogenization on the size properties is the decrease in the dispersion coefficient of size.

Examination of the rheological properties of the emulsions showed a Newtonian behaviour in all cases, agreeing with previous studies (Desrumaux & Della Valle, 1999). However, the viscosity changed with the pressure in the valve (Fig. 4).

It is interesting to see the similarity of behaviour between the curves for the viscosity  $\eta$  and the Sauter diameter  $d_{32}$  (Fig. 4). Both show a complicated behaviour, with four zones, although differences exist in the limits of the zones. In the first zone, up to 90–100 MPa, the Sauter diameter decreased and viscosity increased slowly (Fig. 4). At the same time, the amount of adsorbed protein increased (Fig. 7), as expected, since, as the average droplet size decreases, the specific area increases, which leads to an increase of the fraction of adsorbed proteins. Above 100 and up to ~210 MPa,  $d_{32}$  increased and reached a plateau (Fig. 4), which can be attributed to the 'overprocessing' phenomenon. Simultaneously, the fraction of adsorbed proteins decreased strongly and then increased (Fig. 7). Viscosity followed the same behaviour as  $d_{32}$  (Fig. 4). Above 200–210 MPa the behaviour of the structural and textural properties was complicated and, is probably explained, by the effect of the high pressure on the protein conformation. Indeed, studies on isostatic high-pressure treatment of

proteins have shown that pressure has a huge effect on food protein functionality (Messens *et al.*, 1997). High-pressure effects on proteins are primarily related to the rupture of noncovalent interactions within protein molecules and to the subsequent re-formation of intra- and intermolecular bonds within or between protein molecules (Smith *et al.*, 2000). As extensively described by Messens *et al.* (1997), the whey proteins, particularly the  $\beta$ -lactoglobulin, are far more sensitive to isostatic pressure than other proteins such as BSA. In our case the treatment at high pressure was very short, estimated at  $10^{-4}$  s from the flow rate and the size of the gap ( $\sim 1$   $\mu$ m), but was associated with a significant rise in temperature (Fig. 3). The complicated effects of pressure on emulsions properties could be explained by a change of conformation of the emulsifying whey proteins during the emulsification process. Only a small part of the whey protein is likely to be adsorbed at the water/oil interface in the coarse emulsion, because the kinetics of absorption requires a much longer time than that for emulsification. Thus, a large part of the whey protein was in the aqueous phase during the homogenization and the absorption of the whey protein at the interface occurred after homogenization. In order to understand the possible change of the whey protein, we tested aqueous whey protein solutions (1.5%) before and after a single run in the homogenizer at 300 MPa (Fig. 2). Comparing the  $\mu$ DSC graph with the  $\mu$ DSC graph obtained before treatment in the high-pressure homogenizer (Fig. 2), it can be seen that the endothermic peaks disappeared, indicating that the proteins had probably denatured. This phenomenon is because of the combined

effects of the high shear rate inside the gap (velocity at 300 MPa estimated at  $500 \text{ m s}^{-1}$ ) and the rise of temperature observed. At such a high pressure, it was impossible to slow down the rise of temperature inside the valve, the temperature at the exit being directly proportional to the pressure of homogenization. Electrophoresis measurements of extracted proteins after treatment in the high-pressure homogenizer did not show any significant change in the molecular weights (Fig. 1b); the proteins were not broken down into smaller entities during the homogenization, the time of treatment (estimated at  $10^{-4} \text{ s}$ ) probably being too short.

Over 200–210 MPa, the proteins were denatured and had probably partially lost their emulsifying activity (Fig. 4). The effect of the high-pressure homogenization could be protein aggregation; however, the electrophoresis experiments in the presence of SDS prevents observation of protein aggregation.

## Conclusions

The effects of high-pressure homogenization on oil in water emulsions are complicated. From 20 to 100 MPa, the Sauter diameter decreased, confirming the results of Davies (1985) and Robin *et al.* (1992). Over 100 MPa,  $d_{32}$ , viscosity and the fraction of adsorbed protein displayed up to four zones of behaviour: from 100 to 210 MPa, the Sauter diameter and viscosity increased up to a maximum. This behaviour could be because of the 'overprocessing' phenomenon. Over 210 MPa,  $\mu\text{DSC}$  graphs on whey proteins before and after a single run at 300 MPa in the homogenizer confirmed changes in protein conformation, probably because of the combined effects of high-pressure treatment and the rise in temperature observed. This change in the conformation of proteins probably modifies the emulsifying properties of the whey proteins. There is a strong correlation between the formulation of emulsion and the range of pressure used in homogenization. For sunflower oil in water emulsion (20% oil) stabilized with whey proteins, the optimum pressure of homogenization according to the light scattering measurements is  $\approx 100 \text{ MPa}$ . At this pressure the  $d_{32}$  and the size dispersion coefficient reach a minimum.

## Acknowledgments

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# Microfluidization of dairy model emulsions. I. Preparation of emulsions and influence of processing and formulation on the size distribution of milk fat globules

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**Summary** — The influence of certain process variables (pressure and temperature) as well as composition variables (fat, protein and low molecular weight emulsifier concentrations) on the size distribution of milk fat globules was studied in a dairy model emulsion (oil-in-water) produced by microfluidization, a mechanical emulsification technique. The use of a central composite experimental design allowed us to obtain 2 nonlinear multiple regression equations relating the volume-weighted average diameter of the fat globules ( $\bar{d}_v$ ) as well as the relative size distribution width (cv) to the emulsification pressure (7.9–76.3 MPa) and temperature (35–100 °C), and to sodium caseinate (0.5–3.9 wt%), butter oil (5.2–14.7 wt%) and monoglyceride (0.08–0.88 wt%) concentrations. These 2 functions account respectively for 93.7 and 81.7% of the variation in the average diameter and in the size distribution width of the microfluidized fat globules and were used to explain certain interactions between the different variables affecting the size of the microfluidized fat globules. They were also used to demonstrate the existence of the optimal conditions that correspond to the extremes of the average particle diameter and of the distribution width of the fat globules. Finally, these 2 functions allowed us to predict fat globule size parameters as a function of process and formulation conditions.

emulsification / microfluidization / oil-in-water emulsion / fat globule

**Résumé** — Microfluidisation d'émulsions laitières modèles. I. Préparation des émulsions et influence des facteurs de procédé et de formulation sur la distribution de la taille des globules de graisse. L'influence de certaines variables opératoires (pression et température) et des variables de composition (teneurs en huile de beurre, en protéines et en émulsifiant de faible poids mo-

Symbols used and SI units: [BO] = butter oil concentration (wt%); cv = coefficient of variation or relative standard deviation of the volume-weighted size distribution (%);  $\bar{d}_v$  = volume-weighted average fat globule diameter (m); D = diffusion coefficient (m<sup>2</sup>.s<sup>-1</sup>); F = number of factorial points of the design and Fisher ratio; k = number of independent variables or conductivity of a solution (S.m<sup>-1</sup> = m<sup>3</sup>.kg<sup>-1</sup>.s<sup>3</sup>.A<sup>2</sup>); [MGS] = monoglyceride concentration (wt%); [Prot] = protein concentration (wt%); P = emulsification pressure (Pa = m<sup>-1</sup>.kg.s<sup>-2</sup>); Q = flow rate (m<sup>3</sup>.s<sup>-1</sup>); S = interfacial area (m<sup>2</sup>); T = emulsification temperature (K);  $\alpha$  = distance separating a star point from a factorial point in terms of coded variables (dimensionless);  $\epsilon$  = power density (W.m<sup>-3</sup> = m<sup>-1</sup>.kg.s<sup>-3</sup>);  $\phi$  = volume fraction of the disperse phase (dimensionless);  $\gamma$  = interfacial tension (N.m<sup>-1</sup> = kg.s<sup>-2</sup>);  $\rho$  = mass density (kg.m<sup>-3</sup>).

*lécithaire) sur la distribution de la taille des globules de gras d'une émulsion laitière modèle (huile dans l'eau) a été étudiée pour une technologie d'émulsification mécanique donnée : la microfluidisation. L'utilisation d'un dispositif expérimental central composite a permis l'obtention de 2 équations de régression multiple non linéaires reliant le diamètre moyen des globules de gras ( $\bar{d}_v$ ) ainsi que l'étendue relative de la dispersion des tailles (cv) à la pression (7,8–76,3 MPa) et à la température d'émulsification (35–100 °C), aux teneurs en caséinate de sodium (0,5–3,9% de la masse totale), en huile de beurre (5,2–14,7%) et en monoglycéride (0,08–0,88%). Ces 2 fonctions expliquent respectivement 93,7% et 81,7% des variations du diamètre moyen des globules de gras microfluidisés et de l'étendue relative de la distribution des tailles dans les valeurs considérées des paramètres. Ces fonctions ont en outre permis d'expliquer certaines interactions entre les différentes variables précitées sur la distribution de la taille des globules de gras microfluidisés. Elles ont également permis de mettre en évidence l'existence de conditions optimales correspondant à des extremums du diamètre particulaire moyen et de l'étendue de la distribution de la taille des globules de gras. Finalement, ces 2 fonctions nous ont permis de prédire la distribution de la taille des globules de gras en fonction des conditions de procédés et de formulation.*

*émulsification / microfluidisation / émulsions huile dans l'eau / globules de gras*

## INTRODUCTION

For a number of industries, the homogenization, and more generally the mechanical emulsification of liquids, dietary as well as others, has become over the past many years an important technological process. The use of this type of process in the food, pharmaceutical and cosmetic industries in general and in certain dairy operations in particular is essential for the production of emulsions with certain desirable rheological properties (creams and ointments), textures (ice cream, mayonnaise) and degrees of stability (milk, salad dressing) (Dickinson and Stainsby, 1988). The principle of homogenization and mechanical emulsification processes which, according to Gaulin, must allow "de fixer la composition des liquides" remains simple: in the case of milk, the homogenization process results in a considerable decrease in the size of the fat globules found in the initial suspension, and has the effect of increasing the creaming stability of the emulsion (Tadros and Vincent, 1983). Aside from conventional homogenizers, relatively few mechanical emulsification processes have been and are used in an industrial setting

(Walstra, 1983). During the 1980s, however, other mechanical emulsification processes appeared. One of them, called microfluidization (Cook and Lagace, 1985; Washington, 1987), was initially used principally in the cosmetic and pharmaceutical industries (Chandonnet et al, 1985). More recently, microfluidization was suggested as an alternative method for the production of milk fat microcapsules (Vuillemand, 1991), alcoholized creams (Paquin and Giasson, 1989) and for milk homogenization (Pouillot et al, 1991). If the result of the process is similar to that of homogenization (reduction in the size of the fat globules), the means used to achieve such a result are different. As is the case for conventional homogenization processes, the liquid is forced under high pressure into a chamber. However, in the case of the microfluidization process, the liquid is then divided into 2 microstreams that are projected against one another at high speed and at an angle of 180° (Cook and Lagace, 1985).

Other than a considerable change in the dispersion state of the fat, the emulsification of dietary liquids also results in a considerable rearrangement of the oil-water interface

(Walstra and Oortwijn, 1982) where mainly 2 classes of molecules can be adsorbed: amphiphilic macromolecules (mainly proteins) and low molecular weight emulsifiers (lecithins, monoglycerides, Tweens, Spans, etc) (Dickinson *et al.*, 1990). Proteins and low molecular weight emulsifiers affect the production and stabilization of emulsions. Proteins play 2 major roles: on the one hand they lower surface tension between the interfaces that are formed during the emulsification process, and on the other hand, they form a macromolecular layer surrounding the dispersed particles which structurally stabilizes the emulsion by reducing the rate of re-coalescence (Fisher and Parker, 1986). Low molecular weight emulsifiers affect the production and stabilization of emulsions in several ways: during emulsification, by reducing interfacial tension more rapidly than would the proteins alone, they allow the production of smaller-sized dispersed particles (Darling and Birkett, 1987). Furthermore, while proteins generally stabilize oil-in-water emulsions, low molecular weight emulsifiers tend to destabilize them (Dickinson *et al.*, 1990) by removing protein from the surface of the dispersed particles. This ability of low molecular weight emulsifiers to dislodge macromolecules is due to their greater energy of adsorption compared to individual segments of macromolecules. Finally, if the distribution of these 2 classes of molecules between the surfaces of the dispersed particles and the bulk phase is directly affected by the competitive adsorption between the macromolecules and the emulsifiers at the oil-water interface, it is also a result of the nature of macromolecule-emulsifier interactions at the interface and in the bulk phase (Dickinson, 1986).

Opinions are divided as to the usefulness of using experimental model systems (systems containing a single protein source and/or low molecular weight emulsifier) rather than real food systems (Darling and Birkett, 1987; Dickinson *et al.*, 1990). Even if

the model systems allow relatively precise information to be obtained, they can rarely be extrapolated to real food systems. An alternative would be to use systems that can be called "quasi-foods", that is systems that contain a known mixture of proteins and low molecular weight surfactants and whose concentrations are controlled.

The aim of the study presented here was to examine the influence of certain process (pressure and temperature) and composition (butter oil, protein and surfactant concentrations) variables on the size distribution of fat globules in a model dairy emulsion (oil-in-water) using a given mechanical emulsification technology, *ie* microfluidization.

## MATERIALS AND METHODS

### Experimental design

#### Description

A central composite rotatable design made up of  $k = 5$  factors with 3 levels and 1 repetition was developed. This type of design, the most widely used for fitting experimental results to a second-order model (Pigot, 1986; Box and Drapper, 1987), is called rotatable because the variance of the estimated response  $\hat{y}$  at point  $x$  is uniquely a function of the distance separating this point from the centre point and not of the direction. These designs are composed of  $2^k$  factorial points (usually coded  $\pm 1$ ) with  $2^k$  star points coded  $[(\pm \alpha, 0, 0, \dots, 0), (0, \pm \alpha, 0, \dots, 0), \dots, (0, 0, 0, \dots, \pm \alpha)]$  and  $n_0$  centre points coded  $(0, 0, \dots, 0)$ . The value of  $\alpha$ , on which the rotatability of the design depends, is such that  $\alpha = (F)^{1/4}$  where  $F$  is the number of factorial points of the design: in the present case  $F = 2^k$ . The complex principles for the development of these designs have been presented by various authors including Montgomery (1976), Gacula and Singh (1984), and Box and Drapper (1987).

The design used was therefore composed of  $2^5$  factorial points, with 10 star points (with  $\alpha =$

2.38) and 10 centre points which results in a total of 52 experimental units or emulsions.

The 5 factors that were varied in the preparation of the emulsions are the following: 2 process factors—emulsification pressure (7.8, 20.7, 41.4, 62.1 and 78.3 MPa) and temperature (35, 55, 70, 85 and 100 °C), and 3 formulation factors—protein concentration (0.5, 0.9, 1.5, 2.5 and 3.9 wt%), butter oil concentration (5.2, 8, 10, 12 and 14.8 wt%) and monoglyceride concentration (0.08, 0.2, 0.4, 0.6 and 0.88 wt%).

### Statistical analysis

An analysis of variance was first conducted on the values of the volume-weighted average diameters of the emulsified fat globules ( $\bar{d}_v$ ) and on the relative width (cv) of the particle size distribution. This analysis allowed the split of the total variation of the measured parameters ( $\bar{d}_v$  and cv) according to the principal effects of the treatments, the interactions and the error. The sum of the squares of the differences of the principal effects was split in unitary degrees of freedom using an orthogonal comparison method (Box and Drapper, 1987). This calculation allowed an individual estimation of effects without mutual interference. Two tests were then carried out: a Fisher test allowed a differentiation between the treatments and the interactions which had a significant effect on a given parameter, and an adjustment test (Neter *et al.*, 1985) confirmed that the second-order model allowed an adequate description (to  $\pm 1\%$ ) of the variances in the experimental results.

The effects of the principal treatments and/or interactions which were not significant at the level 95% have been included in the error term. This pooling, which has been justified by Little (1981), increases the degree of significance of the variance due to an increase in the number of degrees of freedom. Although a lower probability threshold could be used in the selection of the effects to be considered in the determination of the regression equation, a probability threshold of at least 95% was used in order to simplify the expression and use of the regression equations.

Finally, following this second analysis of variance, each of the 2 measured parameters ( $\bar{d}_v$  and cv) was analyzed as a function of the principal effects and interactions of process and composition variables. The nullity of the regression

coefficients was tested using Student's test (Neter *et al.*, 1985).

This method for interpreting results is supported and recommended by Little (1981) and Box and Drapper (1987). According to these authors, all the treatment levels in the experimental range are significantly different in their effects after a significant tendency has been established. The best estimates of the effects of the treatments are those values calculated using the regression equation.

### Statistical procedure and graphic

The analysis of variance and regression calculations were carried out using the General Linear Model procedure (Proc GLM and Proc REG) of the SAS (1990).

The 3-dimensional picture of the equations was carried out using Mathematica (Wolfram, 1991).

### Emulsion preparation

#### Ingredients

Sodium caseinates (89.3% protein) were obtained from ICN Nutritional Biochemicals, Canada Ltd (Dorval, Que., Canada). Butter oil (99.4% anhydrous) was purchased from a local dairy cooperative (Agropur, Granby, Quebec, Canada) and was stored at 4 °C. Distilled monoglycerides (EXCEL T-95) with a hydrophile-lipophile balance (HLB) of 4.5 (Shinoda and Kurihara, 1983) were purchased from Alkermix (Bratford, Ontario, Canada). Sodium azide was purchased from Fisher Scientific (Quebec, Que., Canada).

#### Emulsion production

Fifty-two emulsions (600 g) containing 0.5, 0.9, 1.5, 2.5 or 3.9 wt% (based on protein content) sodium caseinates, 5.2, 8, 10, 12 or 14.8 wt% butter oil, and 0.08, 0.2, 0.4, 0.6 or 0.88 wt% monoglyceride were prepared in the following manner: various sodium caseinate solutions, hydrated and solubilized in deionized water ( $k = 1.1 \mu\text{S}\cdot\text{cm}^{-1}$ ) for 90 min, and butter oil, preheated to 50 °C, were mixed using a magnetic stirrer. The solutions were then brought to the appropriate emul-

Table 1. Process variables (protein, butter oil and monoglyceride concentrations, emulsification pressure and temperature) and responses of the central composite rotatable design in terms of volume-weighted average diameter and relative width of the emulsified fat globules distribution.

Variables de procédés (concentrations en protéine, huile de beurre et monoglycéride, pression et température d'émulsification) et réponses en termes de diamètre pondéré en volume et du coefficient de variation de la distribution de la taille des globules de gras émulsifiés.

Trials [Prot] [BO] [MGS] P T ( $\bar{d}_v$ ) cv  
(wt%) (wt%) (wt%) (MPa) (°C) (nm) (%)

#### Factorial points

1	0.5	8	0.2	20.7	55	584	51
2	0.5	8	0.2	20.7	85	584	49
3	0.5	8	0.2	62.1	55	480	58
4	0.5	8	0.2	62.1	85	466	55
5	0.5	8	0.6	20.7	55	427	39
6	0.5	8	0.6	20.7	85	382	38
7	0.5	8	0.6	62.1	55	317	48
8	0.5	8	0.6	62.1	85	326	38
9	0.5	12	0.2	20.7	55	806	69
10	0.5	12	0.2	20.7	85	793	69
11	0.5	12	0.2	62.1	55	808	52
12	0.5	12	0.2	62.1	85	777	53
13	0.5	12	0.6	20.7	55	460	67
14	0.5	12	0.6	20.7	85	481	64
15	0.5	12	0.6	62.1	55	407	47
16	0.5	12	0.6	62.1	85	419	43
17	2.5	8	0.2	20.7	55	509	41
18	2.5	8	0.2	20.7	85	482	47
19	2.5	8	0.2	62.1	55	439	42
20	2.5	8	0.2	62.1	85	358	50
21	2.5	8	0.6	20.7	55	423	41

22	2.5	8	0.6	20.7	85	407	47
23	2.5	8	0.6	62.1	55	381	48
24	2.5	8	0.6	62.1	85	300	51
25	2.5	12	0.2	20.7	55	574	58
26	2.5	12	0.2	20.7	85	583	59
27	2.5	12	0.2	62.1	55	536	52
28	2.5	12	0.2	62.1	85	539	40
29	2.5	12	0.6	20.7	55	809	45
30	2.5	12	0.6	20.7	85	653	46
31	2.5	12	0.6	62.1	55	563	28
32	2.5	12	0.6	62.1	85	509	32

#### Centre points

33	1.5	10	0.4	41.4	70	420	46
34	1.5	10	0.4	41.4	70	427	45
35	1.5	10	0.4	41.4	70	451	45
36	1.5	10	0.4	41.4	70	448	45
37	1.5	10	0.4	41.4	70	415	46
38	1.5	10	0.4	41.4	70	441	46
39	1.5	10	0.4	41.4	70	465	43
40	1.5	10	0.4	41.4	70	433	45
41	1.5	10	0.4	41.4	70	471	41
42	1.5	10	0.4	41.4	70	444	46

#### Star points

43	0.9	10	0.4	41.1	70	442	50
44	3.9	10	0.4	41.4	70	403	29
45	1.5	5.2	0.4	41.4	70	291	31
46	1.5	14.8	0.4	41.4	70	633	52
47	1.5	8	0.08	41.4	70	549	53
48	1.5	8	0.88	41.4	70	324	30
49	1.5	8	0.4	7.8	70	942	39
50	1.5	8	0.4	78.3 <sup>1</sup>	70	461	66
51	1.5	8	0.4	41.1	35 <sup>2</sup>	655	55
52	1.5	8	0.4	41.4	100 <sup>3</sup>	513	58

For the design rotatability to be perfect ( $\alpha = 2.378$ ), the values of some independent parameters should be: <sup>1</sup> emulsion 50,  $P = 90.6$  MPa (in fact, the maximum pressure given by the air feed system was 78.5 MPa); <sup>2</sup> Emulsion 51,  $T = 34.3$  °C; <sup>3</sup> Emulsion 52,  $T = 105.7$  °C.

sification temperature (35, 55, 70, 85 or 100 °C) and the emulsifiers were added. The dispersions, maintained at the emulsification temperature ( $\pm 1.5$  °C), were then mixed for 2 min using a stirrer (Braun CDN Ltd, Model MR7, Mississauga, Ont, Canada). The oil-in-water dispersions were then immediately emulsified using a Microfluidizer (M-110™, Microfluidic Corporation, Boston, MA, USA) at its maximum power setting.

The microfluidization procedure was performed in 2 stages: the first processing was at 7.8, 20.7, 41.4, 62.1 or 76.3 MPa and the second at 4.8 MPa in order to eliminate aggregates formed during the first processing procedure (Ogden, 1973; Walstra, 1975). The composition of the emulsions is given in table 1.

Sodium azide, an antibacterial agent, was added to each emulsion (0.2% vol/vol), and 2 pH

measurements (pH-meter, Coming 140, electrode 476530, Canlab, Montreal, Que, Canada) were carried out.

### Size distribution of the emulsified fat globules

#### Sampling

A sample of each emulsion was taken and stored at 4 °C for 24 h in a 20 ml bottle completely filled. Before analysis, the samples were heated ( $T = 20$  °C) and manually stirred. This procedure, proposed by Walstra (1975), was used in order to prevent a change in the size distribution of the fat globules due to churning.

#### Dissociation of protein structures

The samples were mixed with a buffer (urea, EDTA and  $\beta$ -mercaptoethanol, pH 7) designed by Delgleish *et al* (1987) to dissociate protein structures. Despite the presence of sodium caseinates in the emulsions, the composition of the dissociating buffer was not modified. Measurements were carried out immediately after vigorous manual stirring.

#### Photon correlation spectrophotometry

The size distributions of the fat globules were determined by photon correlation spectrometry (PCS) using the method proposed by Robin and Paquin (1991). The measurements were taken using the Nicomp multifit (7 bit) 64-channel photon correlation system (Pacific Scientific, Hiaz/Royce Instruments Division, Model 370, Menlo Park, CA, USA); the correlation functions were measured on the light that was diffused at an angle of 90° by the particles in suspension. The correlation functions were analyzed by the cumulants method (Koppel, 1972). The first cumulant yielded an average diffusion coefficient ( $D$ ), and the second yielded a mean squared deviation of the average diffusion coefficient ( $\sigma^2$ ). The volume-weighted average particle diameters ( $d_v$ ) were calculated using the Stokes-Einstein law, and polydispersity was expressed by the relative standard deviation or coefficient of variation ( $cv$ ) of the volume-weighted distribution calculated as the distribution width divided

by the average diameter. The accuracy ( $\pm 1\%$ ), the reproducibility ( $\pm 1\%$ ) as well as the length of the analysis ( $2.5 \times 10^6$  photo-impulses or  $\approx 20$  min) were determined on latex sphere solutions (Robin and Paquin, 1991).

Three measurements of the size distribution of the fat globules were carried out on each sample. The values of the average diameters and relative widths of the size distribution of the fat globules are shown in table 1.

#### Average size distributions

A logarithmic transformation was applied to the emulsified fat globule volume-weighted average diameter ( $d_v$ ) and to the homogenization pressure. Indeed, using the theory of isotropic turbulence developed by Kolmogorov (Walstra, 1969, 1983) showed that the maximum size of a fat globule undergoing the homogenization process is a function of the energy density  $\epsilon$  (energy dissipated per unit of volume and time) or of the pressure  $P$  ( $\epsilon = P^{3/2} \rho^{-1/2}$ ), of the interfacial tension  $\gamma$ , and of the mass density  $\rho$  according to:

$$d_{max} = (\epsilon^2 \gamma^3 \rho^{-1})^{1/5} = P^{-3/5} \quad (1)$$

or

$$\log_{10}(d_{max}) \propto -\frac{3}{5} \log_{10}(P) \quad (2)$$

Goulden and Phipps (1964), Walstra (1975) and others have experimentally confirmed these results.

## RESULTS

### Accuracy and reproducibility of the PCS technique

The 10 centre points serve as an indication of the reproducibility of the PCS technique for determining the parameters of the size distribution of the fat globules. These centre points correspond to values obtained from the same emulsion. Using these results, the reproducibility of the evaluation of the  $d_v$  and  $cv$  parameters was approximately 4.2, and

3.2% respectively. However, even though the reproducibility of the results seem satisfying, that is, comparable with those obtained by other methods, their accuracy must be interpreted with some caution. Even though the validity of the PCS technique, in terms of precision and reproducibility, has been demonstrated by scattering from known mixtures of relatively monodispersed polystyrene latex beads (Barger, 1974; Brown and Pusey, 1975), results become less reliable when distributions are larger and polydispersed (Weiner and Techemuter, 1987). Moreover, if the second cumulant is generally dominated by polydispersity rather than by statistical noise in the autocorrelation function, in large distributions the analysis of the cumulant can lead to an underestimation of the variance (Chu and Dinapoli, 1983). Higher order terms should be taken into account, but this remains a difficult problem (Nicol, personal communication).

*Influence of process variables on the average diameter of fat globules and on the distribution width of the diameters*

An analysis of variance was carried out on the values given in table I using orthogonal comparisons between the principal treatments and between the interactions (Box and Draper, 1987). An *F* test was carried out to differentiate the treatments and the interactions which had a significant effect on the volume-weighted average diameter of the fat globules ( $d_v$ ) and on the size distribution of the relative width (cv). An adjustment test of the experimental results for a second-order model was also carried out.

*Average diameter of the emulsified fat globules*

Table II shows the analysis of variance of the  $\log_{10} (d_v)$  as a function of process

Table II. Analysis of variance of the logarithm of the volume-weighted average fat globule diameter versus process and composition variables. *Analyse de variance du logarithme du diamètre moyen pondéré en volume des globules de gras, en fonction des variables de procédé et de composition.*

	df <sup>1</sup>	SS <sup>2</sup>	F <sup>3</sup>	r <sup>2</sup> <sup>4</sup>
Model	20	0.824	38.0 <sup>a</sup>	0.959
Linear effects				
[Prof]	1	0.008	9.6 <sup>a</sup>	
[BO]	1	0.217	251.4 <sup>a</sup>	
[MGS]	1	0.147	170.4 <sup>a</sup>	
$\log_{10} (P)$	1	0.103	119.1 <sup>a</sup>	
T	1	0.006	6.6 <sup>a</sup>	
Quadratic effects				
[Prof] <sup>2</sup>	1	< 10 <sup>-3</sup>	< 0.1	
[BO] <sup>2</sup>	1	0.002	1.9	
[MGS] <sup>2</sup>	1	< 10 <sup>-3</sup>	< 0.1	
$\log_{10}^2 (P)$	1	0.038	43.5 <sup>a</sup>	
T <sup>2</sup>	1	0.025	28.9 <sup>a</sup>	
[Prof] x [BO]	1	< 10 <sup>-3</sup>	0.5	
[Prof] x [MGS]	1	0.004	73.8 <sup>a</sup>	
[Prof] x $\log_{10} (P)$	1	< 10 <sup>-3</sup>	0.4	
[Prof] x T	1	0.001	1.3	
[BO] x [MGS]	1	< 10 <sup>-3</sup>	< 0.1	
[BO] x $\log_{10} (P)$	1	0.006	7.0	
[BO] x T	1	0.002	2.6	
[MGS] x $\log_{10} (P)$	1	0.001	1.4	
[MGS] x T	1	< 10 <sup>-3</sup>	< 0.1	
T x $\log_{10} (P)$	1	0.002	2.0	
Error	31	0.027		
Lack of fit	22	0.002	3.4 <sup>b</sup>	
Pure error	9	0.003		
Total	51	0.851		
Model	8	0.610	80.9 <sup>a</sup>	0.837
Error	43	0.041		
Total	51	0.651		

<sup>1</sup> df = degree of freedom; <sup>2</sup> SS = sum of squares; <sup>3</sup> F = Fisher ratio; <sup>4</sup> r<sup>2</sup> = coefficient of determination. Significance levels: <sup>a</sup>  $p \leq 0.0001$ ; <sup>b</sup>  $p \leq 0.001$ ; <sup>c</sup>  $p \leq 0.01$ ; <sup>d</sup>  $p \leq 0.05$ .

variables. According to the adjustment test, for  $p \leq 0.01$  ( $F < 4.77$ ), the second-order model allowed an adequate description of the variance of  $\log_{10}(\bar{d}_w)$ . Furthermore, the linear effects of all the principal factors were significant (from 95% to 99.999%). Butter oil ([BO]) and monoglyceride ([MGS]) concentrations had a major effect on the variance of the fat globule diameter: they explained 33.4% and 22.6%, respectively, of the total variance. The emulsification pressure ( $P$ ) and temperature ( $T$ ) represented 21.7% and 4.8% respectively, of the variance (the sum of the linear and quadratic effects:  $\log_{10}(P) + \log_{10}^2(P)$  or  $T + T^2$ ) of  $\log_{10}(\bar{d}_w)$ . The effect of the protein concentration was significant ( $p \leq 0.001$ ) but represented only 1.3% of the variance of the  $\log_{10}(\bar{d}_w)$ . The last significant effect given by the analysis of variance was the [Pro]  $\times$  [MGS] interaction which represented 8.8% of the variance of the  $\log_{10}(\bar{d}_w)$ . Other effects were not significant ( $p > 0.05$ ). The error term represented = 4% of the variance of  $\log_{10}(\bar{d}_w)$ .

A regression equation calculated from the significant effects ( $p \leq 0.05$  or more) of the analysis of variance of  $\log_{10}(\bar{d}_w)$  is shown in table III. This second-order model was highly significant ( $p \leq 0.0001$ ) and explains 93.7% of the total variance of  $\log_{10}(\bar{d}_w)$ . All of the regression coefficients were highly significant ( $p \leq 0.0001$ ). It is, however, worth noting that the existence of a minimum average diameter as a function of pressure (regression equation) was not significant, and resulted from an artefact produced by one of the extreme point (star point No 48). A separate regression analysis revealed that the increase in size beyond  $P \approx 50$  MPa (the emulsification pressure from the regression model for which the derivative equals 0) was not significant.

Figure 1 is the response surface obtained from a second-order model relating

Table III. Analysis of regression and second-order model of the logarithm of the volume-weighted average fat globule diameter versus significant variables and interactions.

Analyse de régression et modèle de second ordre du logarithme du diamètre moyen pondéré en volume des globules de gras émulsifiés, en fonction des variables et des interactions significatives.

	Estimate <sup>1</sup>	STD <sup>2</sup>	t
Parameter:			
Intercept	14.293	1.6	8.1 <sup>3</sup>
[Pro]	-0.102	$1.2 \cdot 10^{-2}$	-8.5 <sup>3</sup>
[BO]	0.035	$2 \cdot 10^{-3}$	15.2 <sup>3</sup>
[MGS]	-0.633	$5 \cdot 10^{-2}$	-13.4 <sup>3</sup>
$\log_{10}(P)$	-4.755	0.7	-6.9 <sup>3</sup>
$T$	-0.016	$3 \cdot 10^{-3}$	-5.6 <sup>3</sup>
$\log_{10}^2(P)$	0.506	0.1	6.6 <sup>3</sup>
$T^2$	$1.1 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	5.2 <sup>3</sup>
[Pro] $\times$ [MGS]	0.223	$2.7 \cdot 10^{-2}$	8.2 <sup>3</sup>
$\log_{10}(\bar{d}_w) = 14.293 - 0.102 [\text{Pro}] + 0.035 [\text{BO}]$ $- 0.633 [\text{MGS}] - 4.755 \log_{10}(P)$ $- 0.016 T + 0.506 \log_{10}^2(P)$ $+ 10^{-4} T^2 + 0.223 [\text{Pro}] \times [\text{MGS}]$			
Validity intervals:			
$7800 \leq P \leq 76310 \text{ kPa}$			
$0.5 \leq [\text{Pro}] \leq 3.9 \text{ wt\%}$			
$35 \leq T \leq 100 \text{ }^\circ\text{C}$			
$5.2 \leq [\text{BO}] \leq 14.8 \text{ wt\%}$			
$0.08 \leq [\text{MGS}] \leq 0.88 \text{ wt\%}$			

<sup>1</sup> Estimate = parameter estimated under the regression model (table II). <sup>2</sup> STD = standard error of the estimate.

<sup>3</sup> t = value of Student's Test. Significance level:  $p \leq 0.0001$ .

the values of  $\bar{d}_w$  as a function of the butter oil concentration ([BO], wt%) and of the emulsification pressure ( $P$ , MPa) for a fixed temperature (50  $^\circ\text{C}$ ) and for fixed protein (1.5 wt%) and monoglyceride (0.08 wt%) concentrations. As has been reported elsewhere, the average diameter of the fat globules decreases and reaches a plateau as emulsification pressure increases.



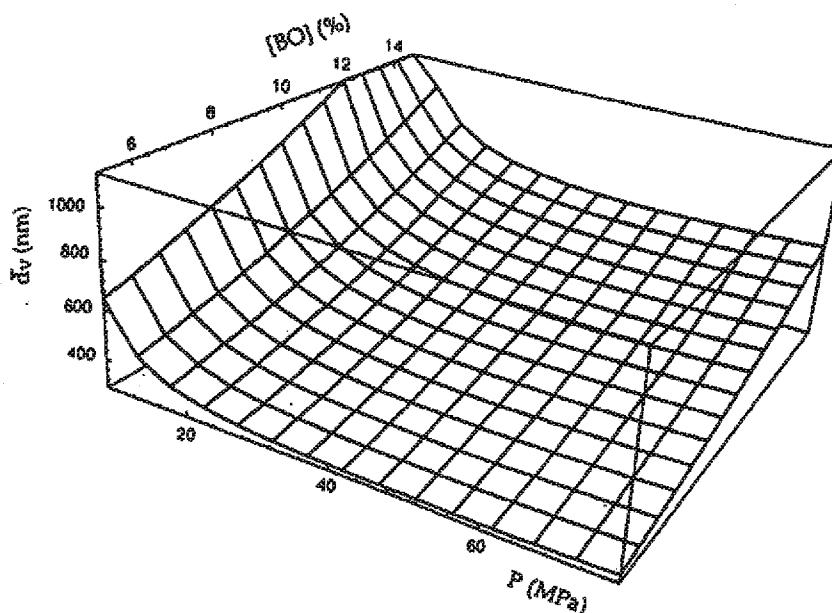


Fig 1. Response surface of the volume-weighted average fat globule diameter as a function of emulsification pressure,  $P$  (MPa), and butter oil concentration,  $[BO]$  (wt%), for a fixed temperature ( $T = 50^\circ\text{C}$ ), a fixed protein concentration ( $[Prot] = 1.5$  wt%), and a fixed monoglyceride concentration ( $[MGS] = 0.4$  wt%).

Surface de réponse du diamètre moyen pondéré en volume des globules de gras en fonction de la pression d'émulsification,  $P$  (MPa), et de la concentration en huile de beurre,  $[BO]$  (%), pour une température donnée  $T = 50^\circ\text{C}$ , et des concentrations fixées en protéines ( $[Prot] = 1.5\%$  et en monoglycérides ( $[MGS] = 0.4\%$ ).

but increases as the fat content increases (Walstra, 1975; Phipps, 1985).

Figure 2 shows the complex influences of emulsifier type and concentration on fat globule size, for emulsions produced under conditions of constant pressure (50.0 MPa), temperature ( $50^\circ\text{C}$ ), and butter oil concentration (10 wt%). Our results are in agreement with previous reports in that, if the presence of a single surfactant results in a dramatic lowering of the size of the fat globules, the near absence of surfactants ( $[Prot]_{\min} = 0.5$  wt%, and  $[MGS]_{\min} = 0.08$

wt%) results in a maximum value for  $\bar{d}_v$  (678 nm).

#### Relative width of the size distribution of the emulsified fat globules

A preliminary statistical analysis has revealed that the variations of  $cv$  as a function of various independent variables could not be adequately described by a second-order model ( $r^2 = 0.71$  after pooling; the adjustment test was not significant) in the original validity interval. A reduction of the

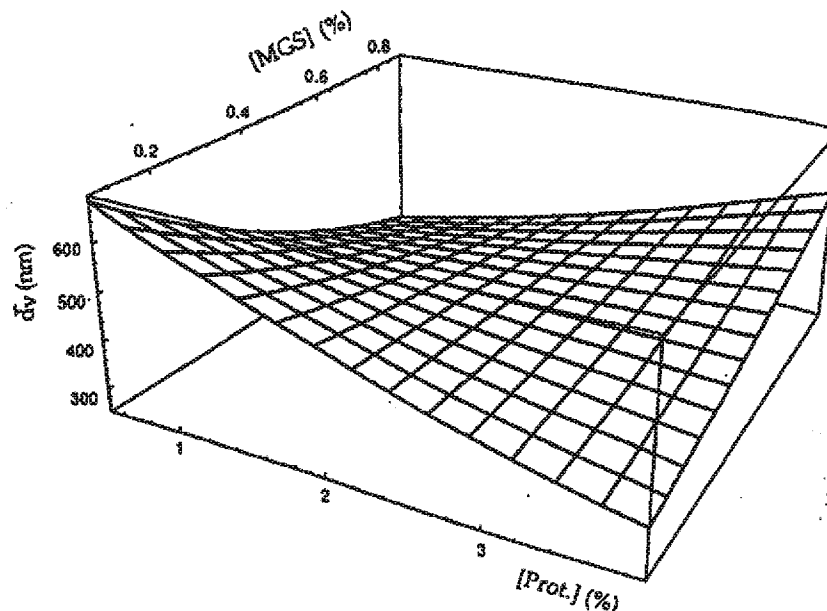


Fig 2. Response surface of the volume-weighted average fat globule diameter as a function of protein,  $[Prot.]$  (wt%), and monoglyceride,  $[MGS]$  (wt%), concentrations for a fixed temperature ( $T = 50^\circ C$ ), a fixed pressure ( $P = 50$  MPa), and butter oil concentration  $[BO] = 10$  wt%.  
 Surface de réponse du diamètre moyen pondéré en volume des globules de gras en fonction des concentrations en protéines,  $[Prot.]$  (%), et monoglycérides,  $[MGS]$  (%), pour une température ( $T = 50^\circ C$ ), une pression ( $P = 50$  MPa), et une concentration en huile de beurre ( $[BO] = 10\%$ ) fixées.

interval corresponding to the elimination of an extreme point (No 49) has consequently been effected.

Table IV shows the analysis of variance of  $cv$  as a function of the process variables. According to the adjustment test, for  $p \leq 0.01$  ( $F < 4.77$ ), a second-order model could adequately describe the variances of  $cv$  in this new validity interval. As mentioned previously, the linear effects of the principal factors, except the temperature one, were significant (from 95% to 99.999%). The protein and monoglyceride concentrations had an important effect on the variance of  $cv$ : they explained respec-

tively 18.0% and 13.4% of the total variance. The emulsification pressure and temperature represented respectively 16.8% and 2.7% (the sum of the linear and quadratic effects:  $P + P^2$  and  $T^2$ ) of the variance of  $cv$ . The effect of the butter oil concentration ( $p \leq 0.0001$ ) represented 7.7% of the variance of  $cv$ . The other highly significant interactions ( $[Prot.] \times [BO]$  and  $[BO] \times P$ ) represented 28.5% of the total variance. The error represented approximately 10.2% of the variance of  $cv$ .

A regression equation, calculated using highly significant effects ( $p \leq 0.0001$ ) of the analysis of variance of  $cv$ , is shown

Table IV. Analysis of variance of the standard deviation of the volume-weighted size distribution of fat globules versus process and composition variables.

Analyse de variance de la déviation standard de la distribution des tailles pondérées en volume des globules de gras en fonction des variables de procédé et de composition.

	df <sup>1</sup>	SS <sup>2</sup>	F <sup>3</sup>	r <sup>2</sup> 4
Model	20	4 198.5	13.1 <sup>a</sup>	0.897
<b>Linear effects</b>				
[Pro]†	1	749.1	46.9 <sup>a</sup>	
[BO]‡	1	381.2	22.6 <sup>a</sup>	
[MGS]§	1	626.7	38.2 <sup>a</sup>	
P	1	156.9	9.8 <sup>a</sup>	
T	1	4.6	0.3	
<b>Quadratic effects</b>				
[Pro] <sup>2</sup>	1	17.9	1.1	
[BO] <sup>2</sup>	1	39.4	2.5	
[MGS] <sup>2</sup>	1	20.8	1.3	
P <sup>2</sup>	1	630.9	39.5 <sup>a</sup>	
T <sup>2</sup>	1	123.9	7.8 <sup>a</sup>	
[Pro] x [BO]	1	359.4	22.5 <sup>a</sup>	
[Pro] x [MGS]	1	23.5	1.5	
[Pro] x P	1	0.2	<0.1	
[Pro] x T	1	75.0	4.7	
[BO] x [MGS]	1	19.7	1.2	
[BO] x P	1	874.8	61.0 <sup>a</sup>	
[BO] x T	1	2.1	<0.1	
[MGS] x P	1	0.5	<0.1	
[MGS] x T	1	7.2	0.4	
T x P	1	3.0	0.2	
Error	30	479.3		
Lack of fit	21	3 592.7	4.1 <sup>c</sup>	
Pure error	9	358.5		
Total	50	4 675.8		
Model	9	3 823.0	20.4 <sup>a</sup>	0.818
Error	41	852.9		
Total	50	4 675.9		

See table II for statistical abbreviations.

Table V. The second-order model, which was highly significant ( $p \leq 0.0001$ ), was able to explain 81.8% of the total variance

Table V. Analysis of regression and second-order model of the variation coefficient of the volume-weighted size distribution of fat globules versus significant variables and interactions.

Analyse de régression et modèle de second ordre du coefficient de variation de la distribution des tailles pondérées en volume des globules de gras, en fonction des variables et des interactions significatives.

	Estimate <sup>1</sup>	STD <sup>2</sup>	t <sup>3</sup>
<b>Parameter:</b>			
Intercept	25.127	18.2	1.4
[Pro]†	12.532	4.1	3.1
[BO]‡	9.477	1.1	8.9 <sup>a</sup>
[MGS]§	-19.278	3.6	-5.4 <sup>a</sup>
P	+2.8 10 <sup>-4</sup>	3 10 <sup>-4</sup>	0.9
T	-1.133	0.4	-2.6
P <sup>2</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	4.1 <sup>a</sup>
T <sup>2</sup>	0.0083	0.03	2.7
[Pro] x [BO]	-1.676	0.4	-4.2 <sup>a</sup>
[BO] x P	-1.3 10 <sup>-4</sup>	2 10 <sup>-6</sup>	-6.8 <sup>a</sup>

$$cv = 25.127 + 12.532 [\text{Pro}] + 9.477 [\text{BO}] - 19.278 [\text{MGS}] - 2.8 \cdot 10^{-4} P - 1.133 T + 10^{-7} P^2 + 0.0083 T^2 - 1.676 [\text{Pro}] x [\text{BO}] - 1.3 \cdot 10^{-4} [\text{BO}] x P$$

#### Validity intervals

$$20\,400 \leq P \leq 76\,300 \text{ kPa} \quad 0.5 \leq [\text{Pro}] \leq 3.9 \text{ wt\%} \\ 35 \leq T \leq 100 \text{ }^\circ\text{C} \quad 5.2 \leq [\text{BO}] \leq 14.8 \text{ wt\%} \\ 0.08 \leq [\text{MGS}] \leq 0.88 \text{ wt\%}$$

See table III for statistical abbreviations.

cv. The value of the regression coefficients of the P term was not significantly different from 0 ( $p > 0.05$ ).

Figure 3, obtained from a second-order model, corresponds to the response surface of the standard deviation of the volume-weighted size distribution of fat globules as a function of emulsification pressure, P (MPa), and butter oil concentration [BO] (wt%), for a fixed temperature

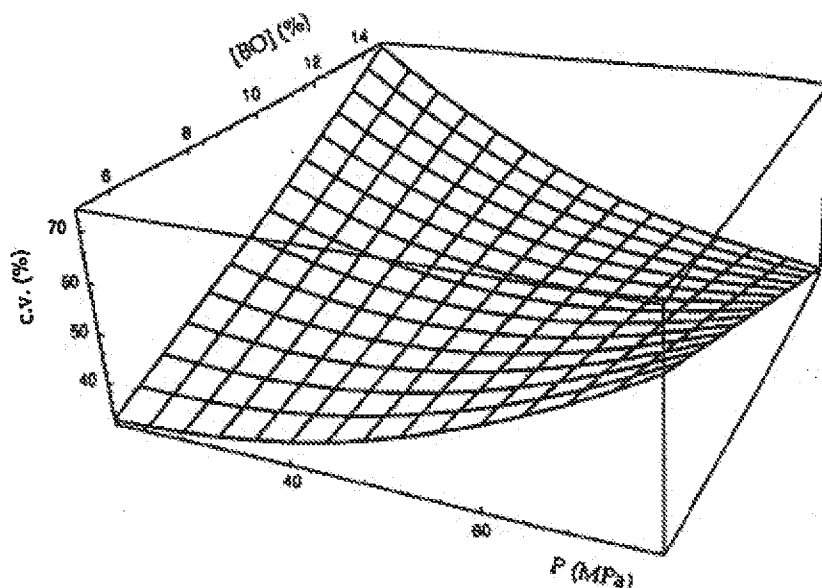


Fig 3. Response surface of the coefficient of variation standard of the volume-weighted size distribution of fat globules as a function of emulsification pressure,  $P$  (MPa), and butter oil concentration [BO] (wt%), for a fixed temperature ( $T = 50^\circ\text{C}$ ), a fixed protein concentration ([Prot] = 1.5 wt%), and a fixed monoglyceride concentration ([MGS] = 0.4 wt%).

Surface de réponse du coefficient de variation de la distribution des tailles pondérées en volume des globules de gras en fonction de la pression d'émulsification,  $P$  (MPa), et de la concentration en huile de beurre, [BO] (%), pour une température donnée ( $T = 50^\circ\text{C}$ ), et des concentrations fixées en protéines ([Prot] = 1.5%) et en monoglycérides ([MGS] = 0.4%).

( $T = 50^\circ\text{C}$ ), a fixed protein concentration ([Prot] = 1.5 wt%), and a fixed monoglyceride concentration ([MGS] = 0.4 wt%). An increase in the emulsification pressure and/or butter oil concentration resulted in an increase in the value of  $cv$ . This figure also shows the minimal values for  $cv$  that are a function of both  $P$  and [BO] (table IV).

Figure 4 shows the response surface obtained from a second-order model of the volume-weighted size distribution of fat globules as a function of protein and

monoglyceride concentration, for a fixed temperature ( $50^\circ\text{C}$ ), pressure (50 MPa) and butter oil concentration (10 wt%). The figure indicates the cooperative effect of surfactant content on the reduction in  $cv$  of this volume-weighted distribution.

#### pH of the emulsions

The average pH of the S2 emulsions was 7.06 with a standard deviation of  $\pm 0.04$ . The emulsions therefore had similar acid-base properties.

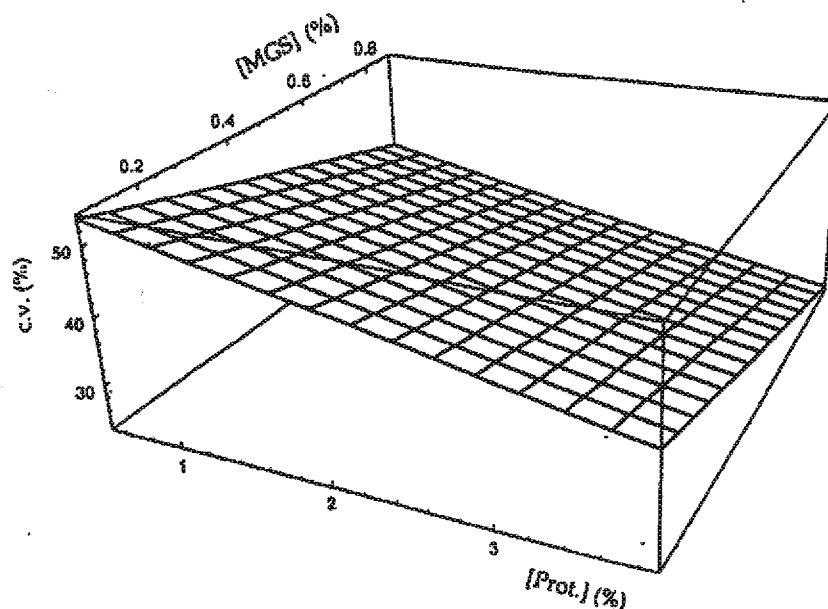


Fig 4. Response surface of the coefficient of variation of the volume-weighted size distribution of fat globules as a function of protein,  $[Prot.]$  (wt%), and monoglyceride,  $[MGS]$  (wt%), concentrations for a fixed temperature ( $T = 50^\circ\text{C}$ ), a fixed pressure ( $P = 50\text{ MPa}$ ), and butter oil concentration ( $[BO] = 10\text{ wt\%}$ ).

Surface de réponse du coefficient de variation de la distribution des tailles pondérées en volume des globules de gras en fonction des concentrations en protéines,  $[Prot.]$  (%), et monoglycérides,  $[MGS]$  (%), pour une température ( $T = 50^\circ\text{C}$ ), une pression ( $P = 50\text{ MPa}$ ), et une concentration en huile de beurre ( $[BO] = 10\%$ ) fixées.

## DISCUSSION

Emulsification is a dynamic process where the disruption and recombination or coalescence of the fat globules take place simultaneously, each with its own rate constant or time scale (Walstra, 1983; Tomberg *et al.*, 1990). Consequently, not only the final droplet size distribution but also other emulsion properties such as rheological behavior will be determined by the conditions leading to an equilibrium between breakdown and coalescence. The

probability of newly formed droplets coalescing depends on the time available for the interfaces to be covered by the surfactants. The time available depends on emulsifying conditions (eg emulsifying machine, power density), which are influenced by the fat content, the ratio of surfactant to fat, and the nature of the surfactants. In order to be active (prevent coalescence), surfactants must not only be transported to the interfaces, they must also be able to adsorb. Adsorption depends on the number of surfactant collisions with fat particles,

and on the probability of the surfactants adsorbing after colliding with the interface. Adsorption is likely influenced by molecular properties such as hydrophobicity, flexibility and charge density (Kato, 1991; Lorient *et al*, 1991). However, the adsorption process occurs on a time scale of less than a millisecond during the homogenization process (Walstra, 1983), so it is very unlikely that an adsorption equilibrium is obtained.

For a given process, the emulsification efficiency is the result of an optimal combination of process variables and of the composition of the liquid to be emulsified. Six major variables are generally considered: emulsification pressure ( $P$ ) and temperature ( $T$ ), liquid flow rate ( $Q$ ), the fraction ( $\phi$ ) of the disperse phase and the surfactant concentration(s) (Walstra, 1983; Phipps, 1985). In the present study, the effect of flow rate on the size distribution of the fat globules was not considered. It has, however, been shown for oil-in-water emulsions (Phipps, 1975, 1982, 1983; Walstra, 1975) and for conventional homogenizers (Manton-Gaulin and/or Rannie), that there is little or no influence of the flow rate of the liquid to be emulsified when  $\phi \leq 30\%$ .

#### *Influence of process variables on the size distribution of fat globules*

##### **Effect of emulsification pressure**

It was empirically shown (Goulden and Phipps, 1984; among others) and then vindicated by Walstra (1968, 1975) that classic homogenization processes (the systems of Manton-Gaulin or Rannie), at moderate emulsification pressures ( $0.25 \text{ MPa} \leq P \leq 40.5 \text{ MPa}$ ), using milk, cream with a fat concentration of  $\phi \leq 12\%$  or any other dilute emulsion, could be quantified by a relation such as  $d = P^m$  where the ex-

ponent  $m$  has a value of  $-3/5$  ( $\{1\}$ ). The exponent  $m$  in the descending slope of the curve  $d_v = f(P^m)$  (fig 1) is of the order  $-0.53 \pm 0.04$ . These results, therefore, seem to corroborate the theories linked to the breakdown of fat globules during turbulent flow conditions. However, there is no general agreement on the functioning of conventional homogenizers at high pressure. According to Walstra (1968, 1983) and Davies (1985) the breakdown of the fat globules can be described by the turbulence theory put forward by Kolmogorov. According to Phipps (1975, 1985), the breakdown of the globules at the entrance to the homogenization valve can be described by the Taylor analysis of shearing forces. The design of the microfluidization chamber (microchannels, mixing zone, etc) differs fundamentally from an homogenization chamber (basically a valve and a seat). Even if the forces that lead to the breakdown of the fat globules are the same, their respective influence on the breakdown is apparently not the same.

Although the convex nature of the curve  $d_v = f(P)_{BC}$  (fig 1) is the result of an experimental artefact and of its statistical consequence (the minimum is very shallow), some authors (Becher, 1967; Phipps, 1975; Tornberg, 1980) have reported that an increase in homogenization intensity ( $E$ ), and/or fat concentration above optimal yields ( $P \geq 40 \text{ MPa}$  or  $E \geq 60 \text{ W}$  and/or  $\phi \geq 12\%$ ) could result in an increase in droplet size. This increase, called overprocessing by Tornberg (1980), should indicate that coalescence is the dominant factor governing the final droplet size of the emulsion. However, the validity of the methods that have been used for producing emulsions and determining average size is questionable. In the present study, it is reasonable to think that an increase in collision frequency with increasing pressure would at most lead to no further decrease in globule size as the minimum is very shallow. An in-

crease in emulsification intensity above optimal conditions, which causes a relatively small change in average droplet size, results in unnecessary energy consumption. The optimal condition corresponded to  $P \approx 50$  MPa. The reduction in fat globule size have various consequences. Emulsions which contain small droplets have a tendency to be more stable with respect to creaming (Stokes' law), and to coalesce more than those containing large droplets (MacRitchie, 1976). According to the MacRitchie approach, which correlates film stability to an energy barrier, the compressional free energy barrier can be, under some conditions, proportional to  $1/d^2$ . However, in practice the effects are not as great as predicted by these equations (Walstra and Oortwijn, 1975; Dickinson and Stainsby, 1982).

If the average particle size can provide some information about the behavior of emulsions, most attention should, however, be paid to the top end of the size distribution as most types of instability are usually first manifested by the behavior of the largest droplets. Although the distribution width of fat globules is an extremely important parameter when characterizing an emulsion, it has not been studied in great depth. Walstra (1975) demonstrated that the relative width of fat globules in homogenized milk sharply increases with the homogenization pressure ( $0 < P < 5$  MPa), reaches a maximum ( $P \approx 5$  MPa) before remaining fairly constant and attaining a limit ( $P \approx 30$  MPa). These results were partly confirmed by Tomberg (1980) studying the behavior of various protein stabilized emulsions made by a sonifier. In the present study, valid for  $20.4 \text{ MPa} \leq P \leq 76.3 \text{ MPa}$ , the results revealed a behavior that differed as a function of butter oil concentration ( $[BO]$ , wt%). At quite high  $[BO]$ , in conformity with previously mentioned results, the relative width of the fat globule distribution diminished as a function of

emulsification pressure. However, at lower concentrations, the behavior was reversed. Although these trends should be interpreted with some caution (lack of accuracy in the determination of the standard deviation, low  $r^2$  value), it appears that trends near central points are similar to those reported elsewhere. At the endpoints of the experimental design, trends are much more difficult to establish, particularly when the decrease in  $cv$  as a function of  $[BO]$  at high  $P$  should arise from the standard deviation(s) being less affected than  $d$ , by the emulsification intensity.

#### Effect of emulsification temperature

Since Gaulin, it has been recognized that the efficiency of the emulsification process is very low in the presence of solid fat (Kessler, 1981). Consequently, the production of oil-in-water emulsions is normally carried out at temperatures above the final melting temperature of the fat ( $T_f \approx 40^\circ\text{C}$ , Jenness and Walstra, 1984). Several authors, studying the homogenization of milk, have shown that an increase of  $10^\circ\text{C}$  in temperature between  $40$ – $70^\circ\text{C}$  decreases the average diameter of the fat globules by 6–8% (Walstra, 1975) to 10–15% (Sweetsur and Muir, 1983). This effect weakens or disappears above  $80^\circ\text{C}$ . As expected, an increase in temperature between  $35$ – $82^\circ\text{C}$  resulted in a decrease in the average diameter of  $\approx 8\%/10^\circ\text{C}$ . Although equation [1] does not predict that the viscosity of either phase can have an effect on the droplet size resulting from the emulsification process in turbulent flow, a decrease of viscosity will understandably affect the rate of passage through the emulsifier and the ease of disruption of the globules. Walstra (1974, 1983) suggested that an increase in the viscosity of the dispersed phase ( $\eta_D$ ) should correspond to a deformation time of a droplet larger than the character-

istic time of an eddy. As the smallest eddies are presumably the most effective (they have the highest kinetic energy), an increase in  $\eta_D$  should lead to a larger spread in flow conditions and consequently in droplet size distribution (cv), as was indeed the case. Moreover, an increase in temperature changes all the composition variables that determine adsorption; fat changes to oil and the macromolecular penetration into the oil becomes possible, hydrophobic interactions at the interface probably become more intense (at least up to 80–85 °C), and the molecular structure of water weakens, which affects its quality as a solvent for surfactants and for hydrophobic interactions. Above 82 °C, the average particle diameter increased slightly. Although this result has not been explained, it should be noted that the influence of the temperature on the average diameter of the fat globules is relatively low: temperature only explains 4.8% (table II) of the total variance of  $\log_{10}(\bar{d}_w)$  and between 82 and 100 °C it represents < 1% which is much lower than the experimental error (= 6.3%). Moreover, the statistical increase in  $\bar{d}_w$  is a consequence of the measurements obtained from an emulsion that was not repeated ( $N = 48$ ) and corresponds to an extreme point (star point) in the experimental design. cv was influenced in a similar manner by the temperature (table V).

#### *Effect of composition variables on the size distribution of fat globules*

##### **Effect of butter oil concentration**

The concentration of fat affects the homogenization efficiency of dairy products when it is > 10% (Walstra, 1975; Phipps, 1985). The microfluidization of the model emulsion demonstrated that an increase in

the butter oil concentration ( $5.2 \leq [BO] \leq 14$  wt%) resulted in a very significant increase in the average diameter of the fat globules ( $\bar{d}_w$ ) (table II, fig 1) and in the size distribution (cv at low pressure) (table III, fig 3). An increase in the fat concentration resulted in a relative decrease in the concentration of surfactant available to cover the new interfacial surface that was formed during the emulsification process. The fat content and the ratio of surfactants to fat affected the extent of coalescence of the newly formed globules by governing the probability that the latter collide before they recover by a surfactant layer. Also, with higher oil concentrations, distances between fat globules decrease and the probability of bridging could increase. This results in a general tendency to increase "particle" diameter.

##### **Effect of surfactant concentration**

The addition of surfactants, which results in a decrease in interfacial tension ( $\gamma$ ), reduces the interfacial free energy ( $\Delta G$ ) of the system,

$$\Delta G = \gamma \Delta S \quad [3]$$

where  $\Delta S$  is the change in the interfacial area

and thereby the Laplace pressure which is beneficial in reducing both the energy requirement to form emulsions and the droplet size that can be obtained. However, this only holds if the ratio (surfactant/interfacial surface) is large enough to cover the new interfacial surface formed during the emulsification process and leading to minimize the coalescence or polymeric bridging (Halling, 1981; Tadros and Vincent, 1983). For minimal protein and monoglyceride concentrations ( $[Prof] = 0.5$  wt%,  $[MGS] = 0.08$  wt%, with  $P = 50$  MPa,  $T = 50$  °C,  $[BO] = 10$  wt%), the average diameter of the fat globules was at a maximum ( $\bar{d}_w = 678$  nm).



Although certain general rules can be deduced from simple thermodynamic principles, the degree and, more importantly, the consequences of the adsorption of protein to the interface can vary considerably with the type of protein and with emulsification conditions. Thus, while Oortwijn and Walstra (1979) reported that whey protein concentration (0.01–2.0%) had relatively little effect on the size of fat globules in homogenized emulsions, Pearce and Kinsella (1978) demonstrated, using homogenized emulsions stabilized with various proteins, that increasing the protein concentration (0.5–5%) could decrease the average diameter of the fat globules by a factor of 2.5. Thus, the nature of the surfactant (hydrophile-lipophile balance, molecular weight, molecular flexibility) as well as its relative concentration (with respect to other surfactants) directly influence the size of the fat globules. Figure 2 illustrates the complex influences of the chemical structure and of protein and monoglyceride concentration on the average diameter of emulsified fat globules; the other variables were kept constant ( $P = 50$  MPa,  $T = 50$  °C,  $[BC] = 10$  wt%). During the emulsification process, smaller droplets ( $d_v = 259$  nm) are produced in the presence of low concentrations of proteins and high concentrations of monoglycerides ( $[Pro] = 0.5$  wt% and  $[MGS] = 0.88$  wt%, respectively) because monoglycerides are better able to lower interfacial tension than proteins alone. With high protein concentrations and low monoglyceride concentrations,  $d_v = 350$  nm. Dickinson *et al* (1989) reported similar trends when studying the effect of octaoxyethylene dodecyl ether ( $C_{12}E_8$ ) with 0.1 wt% caseinate on the droplet diameter of O/W emulsion (20 wt% *n*-tetradecane, pH 7, 25 °C). However, care is needed when analysing these results as the surfactants used were different and hydrocarbons have a higher interfacial tension with wa-

ter than do mixtures of triglycerides with water (Fisher *et al*, 1985).

Moreover, the increase in the concentration of surfactant (for a given  $[BC]$ ,  $P$  and  $T$ ) results in a decrease in the average particle diameter (Dickinson *et al*, 1989) if the monoglycerides to protein ratio is lower or equal to 0.15, eg  $[Pro] = 2.85$  wt% and  $[MGS] = 0.46$  wt% (fig 2). Although these concentrations are independent of the  $[BC]$ , which is rather surprising, it is suggested that an increase in the size of the fat globules above these concentrations can be attributed to,

- a competition between fat and monoglycerides to bind proteins due to the high number of monoglyceride molecules per residue of caseinate and to the formation of hydrophobic bonds between a polar amino acids and the hydrocarbon groups of the monoglycerides (Dickinson and Woskett, 1989). These weak complexes, if they form, should be rather surface-inactive, as the hydrophobic areas of protein and nonionic surfactant bind together, and

- to protein displacement from the interface by monoglycerides. In another study using casein and MGS, Paquin *et al* (1987) found that surface pressure isotherms in the high-pressure region are essentially the same as for the monoglycerides alone, suggesting protein displacement from the interface. It is likely, as suggested by Doxastakis and Sherman (1986), that mono- and diglycerides present in commercial glycerol monostearate form complexes with caseinate at the oil-water interface. It would appear that this (mixed) emulsifier does not behave simply as a nonionic, noninteracting surfactant.

Surfactants (proteins and low molecular weight surfactants) seem to have a cooperative effect on the reduction of  $cv$  (fig 4). As was mentioned in the discussion on the determination of  $cv$ , these results must be interpreted with caution.

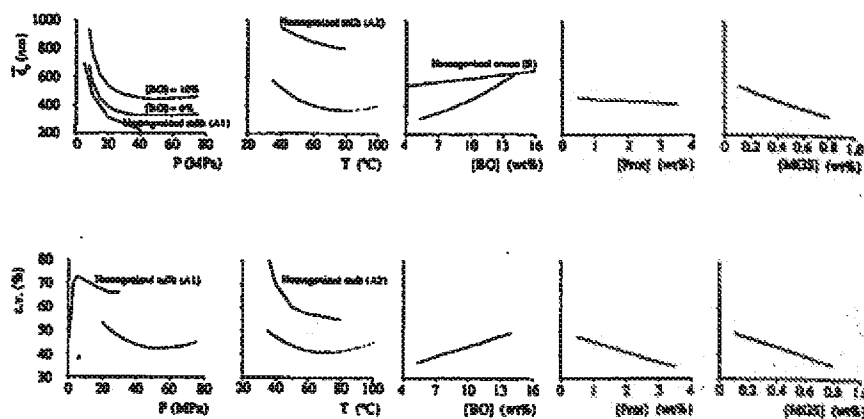


Fig 5. Effect of process and composition variables on fat globule size distribution parameters. For each graph, values of 4 variables were either fixed or specified. Values of fixed variables were:  $P = 50$  MPa,  $T = 50$  °C,  $[BO] = 10$  wt%,  $[Prot] = 1.5$  wt%,  $[MGS] = 0.4$  wt%.

Effets des variables de procédés et de composition sur les paramètres de la distribution des tailles des globules de gras. Pour chaque figure, les valeurs des 4 variables étaient soit fixées soit spécifiées. Lorsque les variables étaient fixées, leur valeur était :  $P = 50$  MPa,  $T = 50$  °C,  $[BO] = 10\%$ ,  $[Prot] = 1.5\%$ ,  $[MGS] = 0.4\%$ .

(A): Walstra, 1975; A<sub>1</sub>, p 282; A<sub>2</sub>, p 289. (B): Phipps, 1985, p 12.

## CONCLUSIONS

The process and composition variables which were studied affect, in varying proportions, the efficiency of the emulsification process called microfluidization. Some of these influences are summarized in figure 5. The variables that had the greatest influence on the size distribution of the fat globules are the butter oil concentration, the concentration of low molecular weight surfactants (monoglycerides), the emulsification pressure, the protein concentration (sodium caseinates) and the emulsification temperature. However, if the average sizes are fairly reliable, the results on relative distribution width must be taken with caution.

The utilization of an experimental design, applied to a dairy-type emulsion, demonstrated the complexity of the influences and interactions (depending on the area of study, pressure, temperature or concentrations) of process and composition variables on the size distribution of the fat globules. If the usefulness of mechanistic and thermodynamic principles is not in doubt, it is still impossible to completely predict all aspects of the behavior of an emulsion.

Finally, although this work was carried out on a model system and although it was demonstrated that for other conditions (if existence of interaction terms) the effect would be different, the results should provide

vide a guide for optimizing the emulsification process.

## ACKNOWLEDGMENTS

We are indebted to the first reviewer for his helpful comments.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Chyi-Cheng CHEN and Bruno LEUENBERGER )

Examiner: L. Channavajjala

Serial No.: 09/726,880 )

Art Unit: 1615

Filed: November 30, 2000 )

For: A VITAMIN POWDER COMPOSITION  
AND METHOD OF MAKING )

DECLARATION OF MR. HERMANN STEIN UNDER 37 C.F.R. §1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Hermann Stein, a citizen and resident of Switzerland, hereby declare as follows:

1. From April 1958 until September 1961 I studied chemical laboratory assistant at Farbwerke Hoechst AG, Frankfurt/Main Hoechst, Germany. From April 1963 until March 1966 I studied chemical engineering at Paul-Ehrlich-Schule, Frankfurt/Main, Germany.

2. Employment history:

- April 1958 - March 1963 chemical laboratory assistant at Farbwerke Hoechst AG
- April 1963 - March 1968 chemical laboratory assistant and chemical engineer at Battelle-Institut e.V., Frankfurt/Main, Germany. Development of new high temperature polymers and new resins for cosmetic applications.

- April 1968 – November 1982 at F. Hoffmann La Roche AG, Basle Switzerland, development of new cosmetic products, starting as head of development laboratory and finally chief of cosmetic development department.
  - December 1982 – January 1984 at Vick International Paris, development of new cosmetic products and know how transfer cosmetic department Hoffmann La Roche to Vick International.
  - February 1984 – January 2002 at F. Hoffmann La Roche AG, Basle, Switzerland and later on Roche Vitamins AG, Basle Switzerland: Development of new vitamin product formulations, improvement of existing vitamin product formulations and development of new production processes for vitamin product formulations. Position: Head of development laboratory and mini plant, process engineer for pilot plant production trials.
  - Since March 2002, I have been a technical consultant for Roche Vitamins AG and DSM.
3. The present application discloses and claims powder compositions containing at least one fat-soluble vitamin dispersed in a matrix consisting of an emulsion-forming composition selected from the group consisting of a natural polysaccharide gum, a mixture of polysaccharide gums, a protein, a mixture of proteins, and mixtures thereof, wherein the fat-soluble vitamin is present in the powder composition in the form of solid droplets having an average diameter of about 80 to about 120 nanometers (nm).

4. I am aware that a Final Office Action issued in the above-identified application on February 23, 2004. I understand that claims 1 and 3-15 were rejected under 35 USC § 103 over Stein *et al.*, EP 0 937 412 ("Stein") or Stein in view of Ford *et al.*, 5,607,707 ("Ford"). (Paper No. 02122004 at 2.) I further understand that claim 17 was rejected under 35 USC § 103 over Stein alone or Tritsch *et al.*, EP 0 841 010 ("Tritsch '010") alone or in combination with Ford in view of Finnan *et al.*, U.S. Patent No. 4,830,859 ("Finnan"). (Paper No. 02122004 at 5.)

5. I am a co-inventor of the subject matter described and claimed in the Stein document relied upon by the Examiner in rejecting the claims of the above-captioned application. Stein is entitled "PREPARATION OF A FINELY DIVIDED PULVEROUS CAROTENOID PREPARATION" and discloses "a continuous process for converting carotenoids, retinoids or natural colourants into finely divided pulverous forms which are particular[ly] required for colouring foodstuff[s] and animal feeds." (Col. 1, paragraph 0001.) In particular, Stein discloses a five step process:

- a) forming a suspension of the active ingredient in a water-immiscible organic solvent optionally containing an antioxidant and/or an oil,
- b) feeding the suspension of step a) to a heat exchanger and heating said suspension to 100-250°C, whereby the residence time in the heat exchanger is less than 5 sec,
- c) rapidly mixing the solution of step b) at a temperature in the range of 20-100°C with an aqueous solution of a swellable colloid optionally containing a stabilizer,
- d) removing the organic solvent and
- e) converting the dispersion of step d) into a pulverous preparation. (Abstract and col. 1-2, paragraph 0008.)

6. During the research that lead to the invention disclosed in Stein, my co-inventors and I, using the knowledge available at the time, attempted to produce the smallest



possible particle size. In particular, Example 5 was included with a view toward optimizing the process by producing the smallest possible particle size. Example 5 is reproduced below:

Solvent: methylene chloride, direct heat transfer (steam).

9.25 kg Fish Gelatin, 18.5 kg of sugar and 2.5 kg of Ascorbyl palmitate were dissolved in 30.25 kg of water in Kettle 1. The pH-value of this matrix was adjusted with NaOH (20%) to 7.2 - 7.6.

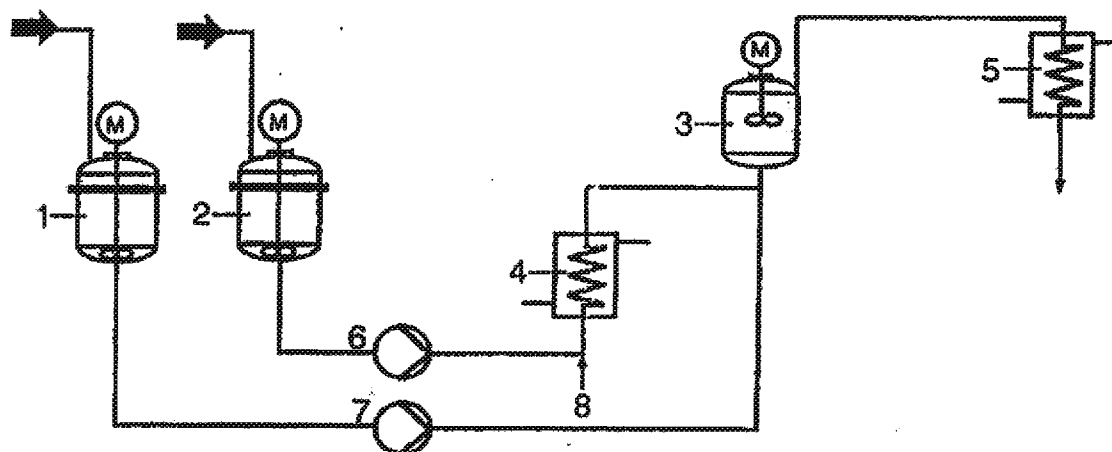
6.0 kg of  $\beta$ -Carotene, 0.75 kg of dl- $\alpha$ -tocopherol and 3.0 kg of corn oil were dispersed in 30.0 kg of methylene chloride in Kettle 2.

The  $\beta$ -carotene suspension was fed by pump 6 with a flow rate of 20 kg/h to the heat exchanger 4 where it was mixed with steam to reach an outlet temperature of 145°C. The residence time in the heat exchanger 4 was 1.3 sec. The matrix was pumped by pump 7 with a flow rate of 30.4 kg/h to the Kettle 3 where the solved  $\beta$ -carotene was mixed with the matrix and emulsified in it. The emulsion was cooled down to 35°C in heat exchanger 5.

Methylene chloride was removed from the emulsion by using a vertical evaporator. The resulting emulsion showed a particle size of the inner phase of 196 nm and was spray dried.

The final product has a  $\beta$ -carotene content of 9.9%, E1/1: 1120,  $\lambda_{\text{max}}$ : 440-460 nm. The powder was well soluble in water, the solution has a very intensive yellow color. (Col. 6, paragraph 0054-0058.)

Figure 1 is reproduced for clarity:



7. As Example 5 shows, at that time, at best we could produce particles sizes of about 196 nm. Based on my unique knowledge of the methods and compositions of Stein, and my long experience in the area of the production carbohydrate matrices, it is my opinion that one of skill in the art at the time of the above-captioned invention familiar with the disclosure of Stein could not have produced particles of the presently claimed size.

8. Moreover, it is also my opinion that one could not have predicted that the process of the present invention would produce significantly smaller particle sizes than the methods of Stein.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: March 16, 2005

Hermann Stein

Hermann Stein